MRC Human Reproductive Sciences Unit

Factors affecting natural antimicrobial expression in the human female reproductive tract.

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Contents

Abstract

The incidence of sexually transmitted infections has increased due to changes in lifestyle and contraceptive practices. The female reproductive tract is therefore increasingly being exposed to potential infection, thus the mechanisms involved in its defence are important. The first line of defence is provided via the mucosal epithelial surface that lines the reproductive tract and the innate immune system. The innate immune response is fundamental to the recognition of infectious agents and the implementation of further defence mechanisms. Natural antimicrobial peptides have been described as being central to the function of the innate immune response. These peptides have been found to be expressed across a wide range of mucosal surfaces, including the reproductive tract. It has been shown that these molecules have cyclical variation providing protective coverage across the menstrual cycle. Their expression appears to be governed by a variety of different effector mechanisms which involve cytokines, sex steroids and cell-cell interactions.

The aims of this thesis are (1) to characterise the expression and regulation of a range of natural antimicrobials within the human endometrium using representative cell lines. (2) To investigate the role(s) played by the interaction between epithelial and stromal cells in the expression of these molecules. (3) To assess the effects of different inflammatory mediators on the production of antimicrobials and the elucidation of the underlying mechanisms. (4) To identify the interactions between antimicrobials and with other innate immune effector molecules. (5) To elucidate the role of sex steroids in the regulation of natural antimicrobials. (6) To examine the expression patterns of these molecules over time in response to infection. (7) To

describe the expression of antimicrobials in human Fallopian tubes with and without an ectopic gestation. (8) To examine the expression of natural antimicrobials in the uterine decidua of ectopic and failed intra-uterine pregnancy.

Mimics of infection such as lipopolysaccharide (LPS – representative of G –ve infection), lipoteichoic acid (LTA – representative of G +ve infection) and cytokines such as IL-1β and TNFα; were demonstrated to alter the mRNA expression of natural antimicrobial molecules in the endometrial cell line Hec-1A (human endometrial carcinoma). The expression of natural antimicrobials was further identified to be both phasic and temporal over time. Changes in expression levels were also observed in primary endometrial stromal cell lines. The interaction between the epithelial and stromal cells concurrently with the addition of mediators of inflammation also yielded a change in mRNA expression. The presence of stromal derived media and mimics of infection caused an increase and earlier expression of molecules such as elafin in the Hec-1A cell line. However, with the addition of progesterone treated stromal derived media, a downregulation of elafin was observed. Further investigation suggested a role for progesterone either directly upon the epithelial cells, which proved to have high levels of genomic progesterone receptors (PR-A and PR-B) or via stromal mediated factor(s). Two such factors were identified and investigated further, TGFβ-1 and MMP-7 (matrilysin). Hec-1A cells treated directly with progesterone demonstrated increased levels of elafin mRNA, however, the presence of stromal cells inhibited this effect. The treatment of Hec-1A cells with TGFβ-1 concurrently with inflammatory stimuli decreased the expression of elafin. However, the addition of MMP-7 increased the expression of elafin mRNA.

In the presence of progesterone endometrial stromal cells up-regulate the level of TGFβ-1 and this has been shown to decrease the epithelial expression of both MMP-7 and elafin.

Antimicrobials are differentially expressed within the Fallopian tube collected during different stages of the ovarian cycle and this appeared to be governed by the circulating levels of the exogenous sex steroids. Many of these molecules were also observed to be increased in response to an ectopic pregnancy. The uterine decidua from an ectopic gestation was also demonstrated to show differential expression to that observed in the decidua of both surgically managed miscarriage and from termination.

This work furthers our understanding in the expression and regulation of antimicrobials in the upper reproductive tract, including the role of paracrine mediated factors. The altered expression in the presence of an ectopic gestation both in the Fallopian tube and uterine decidua indicates that further research may offer preventive, diagnostic and treatment opportunities in adverse pregnancy outcomes.

Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Donna Marie Dalgetty

Dedication

This thesis is dedicated to the memory of my father, David Dalgetty whose early encouragement of my inquisitive nature and shared enthusiasm for the how and why of things has fostered my love of science. This was our dream and although not here to share it, he provided me with the strength and determination which has served me so well, his words remain within my head and heart. Dad – we made it!

"Our lives are not determined by what happens to us but by how we react to what happens, not by what life brings to us, but by the attitude we bring to life. A positive attitude causes a chain reaction of positive thoughts, events, and outcomes. It is a catalyst, a spark that creates extraordinary results. "

Anon

Acknowledgements

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Abbreviations

ABC	Avidin biotin complex
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine – 3', 5' - monophosphate
cDNA	Complementary DNA
COCP	Combined oral contraceptive pill
COX 1/2	Cyclo-oxygenase – 1/2
CRE	cAMP response element
DAB	3, 3' – diaminobenzidine
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EP	E prostanoid receptor (prostaglandin E receptor)
ER	Oestrogen receptor
FAM	6 – carboxyfluorescein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluoroscene isothiocyanate
FSH	Follicle stimulating hormone
G/M-CSF	Granulocyte/macrophage colony stimulating factor
hBD	Human beta defensin
HLA	Human leukocyte antigen
HNP	Human neutrophil defensin
HSP	Heat shock protein
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3 – dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of NFκB
IL	Interleukin
IL-1ra	Interleukin - 1 receptor antagonist
IL-1R1/11	Interleukin – 1 receptor type 1/11
IP-10	IFNγ inducible protein – 10
IRAK	IL-1 receptor associated kinase
LBP	Lipopolysaccharide binding protein
LF	lactoferrin
LIF	Leukaemia inhibitory factor
LH	Luteinising hormone
LMP	Last menstrual period
LNG-IUS	Levonorgestrel-releasing intrauterine system

LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAL	MyD88 – adaptor – like protein
MCP-1	Monocyte chemotactic protein – 1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NBF	Neutral buffered formalin
NCAM	Neural cell adhesion molecule
NF IL-6	Nuclear factor interleukin – 6
NFκB	Nuclear factor kappa B
NK	Natural killer
P4	Progesterone
PAMP	Pathogen asscociated molecular pattern
PBS	Phosphate buffered saline
PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase
PGES	Prostaglandin E synthase
PGFS	Prostaglandin F synthase
PID	Pelvic inflammatory disease
Poly I:C	Polylinosine – polycytidylic acid
PR	Progesterone receptor
PRR	Pathogen recognition receptor
Q – RT – PCR	Quantitative – reverse transcription – polymerase chain reaction
RANTES	Regulated upon activation, normal T cell expressed
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
rRNA	Ribosomal RNA
RU486	Mifepristone
s.e.m	Standard error of the mean
SLPI	Secretory leukocyte protease inhibitor
STAT	Signal transducer and activator of transcription
STI	Sexually transmitted infection
TAMRA	6 - carboxytetramethylrhodamine
TCR	T cell receptor
TGF	Transforming growth factor
Th 1/2	T helper 1/2
TIR	Toll/IL-1 receptor homology
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPA	Tissue plasminogen activator
TRAF	TNF receptor associated factor
uNK	Uterine NK
VCAM-1	Vascular cell adhesion molecule – 1
VEGF	Vascular endothelial growth factor

University of Edinburgh

Chapter 1:

General Literature Review

Chapter 1: General Literature Review

1.1 The Female Reproductive Tract

1.1.1 The human uterus and the menstrual cycle

The uterus is the organ responsible for maintaining the viability of the fetus and providing support for the duration of pregnancy. Within the uterus there is a lining of glandular tissue, the endometrium, which is made up of a basal and functional layer. In the absence of pregnancy the human endometrium responds to cyclical changes involving the sequential degeneration and regeneration of the functional layer. These changes occur over an average 28 day period and are responsive to the sequential exposure of the ovarian hormones, oestrogen and progesterone. The maturation of the endometrium is essential in the preparation for the implantation of the blastocyst. The uterine cycle consists of three phases consisting of menstrual flow, proliferation and the secretory phase (figure 1.1.1 depicting the cyclical changes of the endometrium). The classic histological features are described by Noyes (Noyes, Hertig et al. 1950).

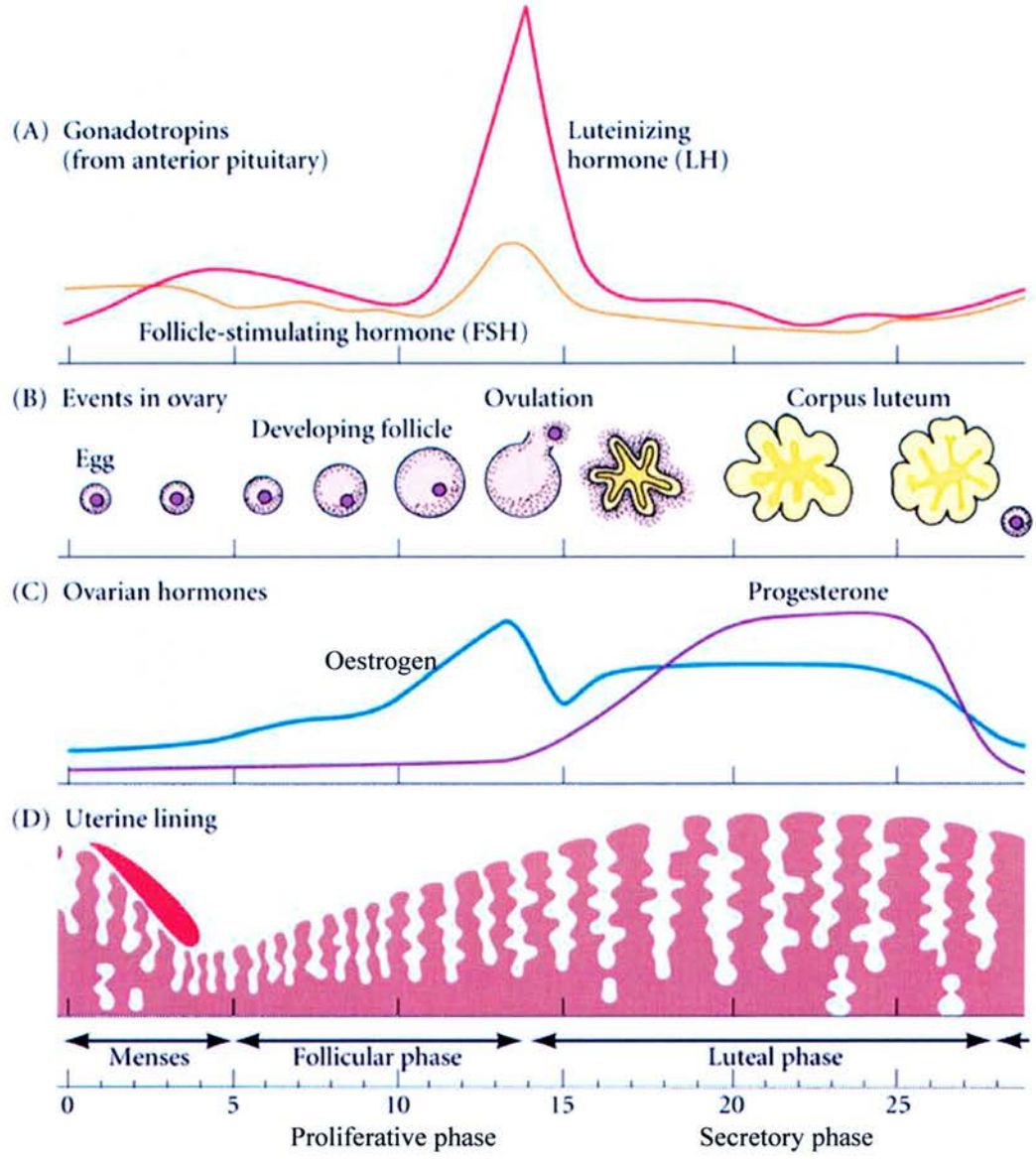


Figure 1.1.1 (A) Concentrations of pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH). (B) Events in the ovary related to the ovarian cycle with follicle selection, maturation and ovulation. This is followed by formation of the corpus luteum, which then regresses. (C) The levels of ovarian hormones, oestrogen (blue) and progesterone (purple) in the circulation across the menstrual cycle. (D) The cyclical changes within the endometrium showing the changes in the uterine lining. (Figure adapted from (Gilbert 2006)).

1.1.2 Regulation of the menstrual cycle

The process of menstruation occurs when implantation fails to occur and there is luteal regression, progesterone withdrawal and thus the functional layer of the endometrium is shed. This occurs in response to the demise of the corpus luteum and the resultant decrease in the levels of progesterone. Much of our current understanding of the vascular changes during menstruation comes from the work of Markee on the rhesus monkey in 1940 (Markee 1940). This work also determined that the steroid hormones are the major factors in the control of these structural changes. There are also vasoactive substances in the menstrual fluid in the form of prostaglandins, PGE_2 and $PGF_{2\alpha}$ (Lumsden, Kelly et al. 1983). $PGF_{2\alpha}$ is a vasoconstrictive substance and it has been suggested that this agent is pivotal to the start of menstruation (Baird, Cameron et al. 1996). Explant studies have implicated hormones in the regulation and production of these prostaglandins (Abel and Baird 1980; Abel and Kelly 1983). There is also an increase in prostaglandin dehydrogenase (PGDH), the enzyme that mediates the metabolism of PGE_2 and $PGF_{2\alpha}$, indicating progesterone dependence of this enzyme (Casey, Hemsell et al. 1980; Critchley, Wang et al. 1998; Hapangama, Critchley et al. 2002).

The menstrual phase is also considered to be an inflammatory event (Finn 1986; Kelly, Illingworth et al. 1994). In the pre-menstrual phase, leukocytes increase in number in the endometrium; this provides a level of immune defence as well as mediating the breakdown of tissue. In addition, a subset of NK cells, characterised by the expression of the CD56 surface marker, increase in number from mid-cycle onwards. Leukocytes release proteases and stimulate the release of cytokines and

chemokines, the key mediators of the inflammatory response. The leukocytes include macrophages, neutrophils and uterine natural killer (uNK) cells, most of which lack classic oestrogen receptor α (ERα) and progesterone receptors (PR) (Poropatich, Rojas et al. 1987; Henderson, Saunders et al. 2003). The uterine natural killer cells have been shown to express oestrogen receptor β (ERβ) and the glucocorticoid receptor (GR) (Henderson, Saunders et al. 2003). This lack of ERα and PR is indicative of a degree of paracrine mechanism of control over these cells; oestrogen could act directly via ERα and cortisol via GR. Neutrophils are present throughout the cycle at a basal level, with a dramatic increase observed in the premenstrual phase at regions of uterine inflammation (Noyes, Hertig et al. 1950; Kamat and Isaacson 1987; Poropatich, Rojas et al. 1987). This migration of neutrophils may be mediated via the chemotactic factor interleukin – 8 (IL-8) (Rampart, Van Damme et al. 1989; Colditz, Zwahlen et al. 1990).

1.1.3 Oestrogen and Progesterone

The co-ordinated actions of the steroid hormones oestrogen and progesterone are central to the regulation of the female reproductive system. Oestrogen and progesterone have significant roles throughout the reproductive tract, however their actions have also been observed in other systems, such as bone remodelling and peripheral vasoreactivity (Prior 1990; Hashimoto, Akishita et al. 1995). There have also been many reports supporting a role for steroid hormones and their receptors in immunomodulation. Monocytes and macrophages have been shown to express nuclear ERs by a number of independent groups (Ashcroft, Mills et al. 2003; Carruba, D'Agostino et al. 2003). The presence of these receptors may allow sex

steroids to directly or indirectly participate in immune regulation; such as the differentiation of monocytes and cytokine release from the macrophages (Ashcroft, Mills et al. 2003; Carruba, D'Agostino et al. 2003; Capellino, Montagna et al. 2006). Whilst other studies have shown that there is a role for oestrogens in chronic inflammatory conditions such as rheumatoid arthritis (Cutolo, Sulli et al. 1995) and endometriosis (Giudice and Kao 2004). Gender-based differences in response to inflammatory conditions have also been attributed to immuno-modulation by the sex steroids (Wira, Fahey et al. 2005). The immune system of the female reproductive tract is hormonally influenced and provides protection from infection throughout the menstrual cycle. The modulation of cytokines, leukocytes and the interactions between the innate and adaptive immune systems are critical to the maintenance of an infection free environment and for the support of conception (Wira, Fahey et al. 2005).

Oestrogen acts specifically on the uterus during the proliferative phase, bringing about both the proliferation and importantly the priming of progesterone receptors (PRs). Oestrogen and progesterone are ligands and both act upon the nucleus and whilst the receptor controls the action of the hormones, the hormones are also able to mediate the level of receptor expression. When the receptors bind their hormone, gene transcription is modulated to bring about a change in function. The oestrogen receptor (ER) has two transcribed forms, ERα (Greene, Gilna et al. 1986) and described a decade later, ERβ (Kuiper, Enmark et al. 1996; Mosselman, Polman et al. 1996). Currently, there are also three main isoforms of the progesterone receptor (PR), designated simply as PR-A, PR-B and PR-C. Both of the PR-A and PR-B

isoforms are expressed from different promoters on the same gene (Kastner, Bocquel et al. 1990; Mote, Arnett-Mansfield et al. 2006). The isoforms are virtually identical, with PR-B exhibiting an additional 164 amino acids, at the N-terminal end. When the receptor binds with progesterone, it undergoes a conformational change and undergoes dimerisation. The third isoform is PR-C, which is believed to have a role in the regulation of the transcriptional activity of PR-A and PR-B (Wei, Gonzalez-Aller et al. 1990; Wei, Hawkins et al. 1996). Recently, it has also been demonstrated that the PR-C isoform is upregulated at term and in labouring myometrial cells and may serve to inhibit endogenous levels of PR-B transcriptional activity (Condon, Hardy et al. 2006). PR-C does not have DNA binding capacity, but, can bind to progesterone (Wei, Norris et al. 1997) and render it unavailable for PR-B. There is also a fourth PR (PR-M), which has only been identified recently and is still to be further characterised. It has been described as a truncated form and was cloned from human adipose and aortic cDNA (Saner, Welter et al. 2003).

There is crosstalk between the ER and PR, with oestrogen having control over the PR as well as its natural progesterone ligand. A number of experiments utilising rat uterine cells have demonstrated that when progesterone receptors are bound with progestin or anti-progestin, the result is an inhibition in oestradiol stimulation of the ER (Katzenellenbogen 2000). It has also been shown that the use of the intra-uterine contraceptive device, levonorgestrel-releasing intrauterine system (LNG-IUS), which releases a high dose of progestin, downregulates the ER, PR-A and PR-B. This downregulation is observed in both the glands and the stroma of the endometrium,

thus further indicating the requirement for progesterone and its receptors for the maintenance of ER levels (Critchley, Wang et al. 1998).

1.1.4 The proliferative phase and secretory phase of the menstrual cycle

Proliferation occurs from day 4 to day 14 in the menstrual cycle and is regulated by oestrogen. Both oestrogen and progesterone receptors are expressed within the stromal and glandular compartments (Lessey, Killam et al. 1988; Snijders, de Goeij et al. 1992; Wang, Critchley et al. 1998). Oestradiol is a product of the developing Graaffian follicle. The pituitary hormone FSH mediates the maturation of at least one of these follicles, stimulating the release of oestrogen (oestradiol), which in turn stimulates the proliferation of the endometrium. Oestrogen stimulation of the endometrium gives rise to a number of different changes, such as the formation of new glands and stromal regeneration from the residual basal layer. The formation of new glands is characterised with the increased prominence of the nuclei which are undergoing mitosis. There is also an increase in mitosis within the compact stromal compartment. At the end of this phase, "day 14", there is a huge increase in the levels of the pituitary hormone LH, consequent upon increasing oestradiol levels from the developing follicle prior to ovulation.

The secretory phase occurs conventionally from day 16 to 25 and is mediated via the steroid progesterone. LH also continues to be released from the pituitary and is responsible for the luteinisation of both the theca and the granlulosa cells of the corpus luteum. The granulosa lutein cells are responsible for the secretion of

progesterone. These events combine in maintaining the secretory transformation of the endometrium.

During the early secretory phase the glands develop sub-nuclear vacuoles of glycogen and become convoluted in appearance. The level of mitosis within the glands begins to decrease and ceases around day 18. Also at this time the glycogen deposits can be observed on the apical side of the cells and are maximally released around days 20 – 21, until depleted. At the mid to late secretory phase, the tortuosity of the glands is increased, whilst the stromal oedema increases for the duration of the secretory phase, there is a peak in the mid-secretory phase. The stromal cells change both structurally and functionally during this mid to late secretory phase. These pre-decidual changes begin at the spiral arterioles and occur across the whole of the stromal region. Decidualisation of the stromal cells occurs in preparation for blastocyst implantation and progressively forms the decidua during pregnancy (Noyes, Hertig et al. 1950). Pinopodes are expressed between days 19 to 21 on the apical surface of the luminal uterine epithelium. This process is progesterone dependent and their appearance corresponds with the likely time of implantation (Martel, Monier et al. 1991; Nikas, Drakakis et al. 1995; Nikas and Psychoyos 1997).

In the late secretory phase, leukocytes infiltrate the endometrium with both macrophages and lymphocytes being represented (Bulmer, Longfellow et al. 1991). However, the predominant leukocyte is the uterine natural killer (uNK) cell. These cells are abundant during this phase and remain through the first trimester of pregnancy (King, Wellings et al. 1989; Bulmer, Longfellow et al. 1991). The

significance of this, and particularly with relevance to the immuno-modulation of the female reproductive tract, will be discussed in more detail throughout this thesis.

1.1.5 Sex steroid receptor expression across the menstrual cycle

There has been extensive immunohistochemical investigation of the human endometrium in the elucidation of cyclical variation of ER and PR levels and their occurrence within both the basal and functional regions, within both the epithelial and stromal compartments of the endometrium (Garcia, Bouchard et al. 1988; Lessey, Killam et al. 1988; Critchley, Bailey et al. 1993; Snijders, de Goeij et al. 1996; Wang, Critchley et al. 1998). All of these studies have demonstrated the nuclear localisation of both receptors. PR-A and PR-B have been located within the glands and the stroma in the proliferative phase. However, during the secretory phase onto early pregnancy, it is only the PR-A isoform that is observed and this is restricted to the nuclei of the stromal cells. This indicates that the PR-A is likely responsible for the action of progesterone in the luteal phase within the stroma and specifically decidualisation. The immuno-expression of ERα has been shown to increase in the endometrial functional layer during the proliferative phase peaking late on in the phase. However, the ER in the glands is downregulated during the secretory phase, and this is in response to the increasing levels of progesterone. This effect can be inhibited with the administration of the anti-progestin, mifepristone (RU486), early in the secretory phase (Maentausta, Svalander et al. 1993; Kumar, Zhu et al. 1998). There is a decline in the levels of ERα in both the glandular and stromal compartments throughout the secretory phase. ERβ mRNA was detected in both the glands and the stroma, however, there was a decrease in expression within

the glands in the late secretory phase (Critchley, Brenner et al. 2001; Lecce, Meduri et al. 2001). ERβ has also been shown to be expressed in endothelial cells, which may imply an influence on the regulation of vascularisation. Women with compromised fertility treated with clomiphene citrate, which is an anti-oestrogen, demonstrated lower pregnancy rates. This correlated with the low levels of oestrogen receptors within the pre-ovulatory endometrium (Ohno and Fujimoto 1998).

In-vitro it has been shown that PR-A is more prevalent than the PR-B isoforms during decidualisation. However, there is marked reduction in the availability of PR-A as decidualisation progresses. This reduction is further accelerated with the addition of a synthetic progestin (Brosens, Hayashi et al. 1999). Also, the transient transfection of either of the PR isoforms brought about an inhibition of decidual prolactin (PRL) promoter-reporter construct in response to cAMP. The insertion of an LNG-IUS brings about a downregulation of the PR isoforms within both the glands and the stroma (Critchley, Wang et al. 1998). This suggests that the regulation of both receptor isoforms is mediated by their ligand. However, it is suggested from murine models, that it is PR-A which is essential to the function of the uterus and the ovaries (Mote, Arnett-Mansfield et al. 2006). It was ascertained that in the chick oviduct PR-A and PR-B are translated from two separate start codons within the same gene. This provided the opportunity to knock out either one of the isoforms in order to determine the roles of the respective isoforms. The importance of functioning PRs was demonstrated to be crucial to fertility with the use of knockout mice. These mice were rendered infertile, with specific dysfunction

in ovulation, implantation and the decidualisation of stromal cells (Mote, Arnett-Mansfield et al. 2006). In the mouse PR-A knockout, there was infertility, whilst the PR-B knockouts proved to be essential to the morphology of the mammary glands (Mote, Arnett-Mansfield et al. 2006). In contrast to the human where the activities of the two isoforms are mediated by their co-localisation, the expression of PR-A and PR-B in the mouse may be separate (Mote, Arnett-Mansfield et al. 2006).

1.2 Immunity

The human body is constantly exposed to a myriad of infectious agents, and due to the effectiveness of our immune systems this rarely manifests as an infection. The mammalian immune system has been classically divided into two distinct parts; the innate immune response and the adaptive immune response. The innate immune system is the first line of defence, and has been described as primitive by some. Whilst the adaptive immune response acts in synergy and in response to signals that are received from the innate effectors, and is second in line, but, serves as the more 'experienced' component.

1.2.1 Adaptive immunity

The adaptive immune response is acquired and is distinct from that of the innate immune response because of its specificity. A number of different molecules and effectors are involved which interact directly with specific antigens. Adaptive immunity can be further divided into that of a humoral or cellular mediated response.

Humoral immunity is directed towards extracellular antigens and the B-cells are the main components. B-cells are stimulated upon the recognition of an antigen or they can be activated by dendritic cells. This activation brings about a differentiation into a plasma cell, and this begins to produce an antibody which is specific against the target antigen (Veeraswamy, Cella et al. 2003).

Dendritic cells (DCs) are members of the innate immune system; however, they are pivotal to the function of the adaptive response. Dendritic cells have often been described as the 'bridge' between the two components of immune defense (Clark and Kupper 2005) (figure 1.2.1.1). DCs serve as antigen presenting cells (APCs), and control and initiate the immune response through the activation of naïve T cells (Banchereau and Steinman 1998). Immature DCs are localised within peripheral non-lymphoid tissues and upon inflammation or infection, they become mature with an upregulation of MHC class II molecules (Banchereau and Steinman 1998; Pulendran, Palucka et al. 2001; Banchereau 2002). The DCs migrate to secondary lymphoid tissues and become terminally differentiated in the T-cell areas (Cella, Scheidegger et al. 1996; Cella, Sallusto et al. 1997). Mature DCs (mDCs) produce cytokines including IL-12 which is involved with the production of IFN-γ-producing T-helper 1 (Th1) cells (Macatonia, Hosken et al. 1995). The culture of peripheral blood monocytes with IL-4 and GM-CSF (granulocyte-macrophage colony stimulating factor) can be differentiated into immature DCs (Banchereau and Steinman 1998).

Cellular immunity is directed towards intracellular antigens such as that of viral particles. The key mediators of cellular immunity are the T-cells, which utilise the T-cell receptor (TCR) in the recognition of pathogens. The TCRs recognise foreign peptides on the surface of cells by the major histocompatibilty complex (MHC). When a foreign antigen is presented and recoginised by the T-cell, a subset of T-cells are capable of directly killing that cell. After activation both B- and T- cells undergo clonal expansion which serves to increase the response (Janeway, Travers et al. 2001).

The adaptive immune response is responsible for the memory of the encountered pathogens ensuring a rapid response in the event of re-infection. The adaptive immune response typically takes 3-4 days to gain the upper hand over a microbial infection. This delay in response would provide the average microbe with a doubling time of approximately 20 minutes, a huge advantage over the advancing immune defence molecules (Janeway, Travers et al. 2001). Thus, it would be unlikely that we would survive with the reliance solely upon the adaptive immune response; hence the more rapid response of the innate response has a crucial role to play in host defence.

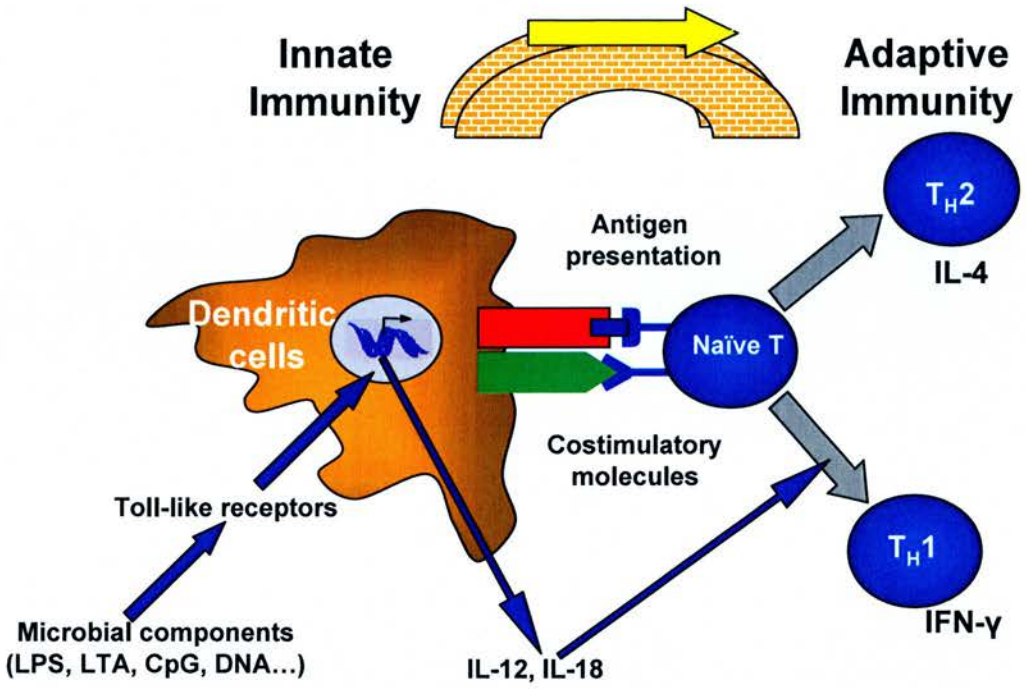


Figure 1.2.1.1 A simplified representation of some of the interactions of dendritic cells at the interface between the innate and adaptive immune response.

1.2.2 Innate immunity

The innate immune system serves as the first line of defence and as such is in a state of constant activation. The main cell components of the innate immune system are that of the epithelium and phagocytes. The epithelium represents the outer barrier or first line of defence from the environment (Janeway, Travers et al. 2001). This outer layer of epithelium functions as a mechanical barrier and is responsible for the production of a host of effector molecules of the immune response (Boman 2000). Amongst these molecules are the microbicidal small peptides – natural antimicrobials. Natural antimicrobial peptides are secreted by epithelial cells both in response to infection and/or constitutively (Ganz 1987). They are also produced by

phagocytic cells such as the neutrophilic granulocyte cells, which function to engulf any invading microbe and are amongst the first cells to arrive at the site of infection. Some antimicrobials also function as antiproteases or are chemotactic for other immune effector cells, thereby enhancing the response (Territo, Ganz et al. 1989; Chertov, Michiel et al. 1996).

The innate immune response was previously thought not to be as specific as the adaptive, and there are no memory cells, nor is clonal expansion involved. However, it has become increasingly apparent that there is more specificity than has traditionally been realised. The innate immune response is triggered in response to common structural patterns exhibited by microbes, known as pathogen-associated molecular patterns (PAMPS) (Medzhitov and Janeway 2000). Innate immune cells express pattern recognition receptors (PRRs), which enable the recognition of pathogens. Among these receptors are a family known as the toll like receptors (TLRs) (Akira and Sato 2003; Zhang, Zhang et al. 2004). These receptors are responsible for the recognition of different pathogens; such as proteoglycans (TLR2) (Takeuchi, Hoshino et al. 1999), single stranded RNA (TLR7) (Lund, Alexopoulou et al. 2004; Yang, Puel et al. 2005; Hoshino, Sugiyama et al. 2006), bacterial DNA (TLR9) (Hemmi, Takeuchi et al. 2000), LPS (TLR4) (da Silva Correia, Soldau et al. 2001), Flagellin (TLR5) (Hayashi, Smith et al. 2001), double stranded RNA (TLR3) (Alexopoulou, Holt et al. 2001). A microbe approaching a cell exhibiting one or more of these patterns will result in a signalling cascade via MyD88 (myeloid differentiation factor 88) and TIR domain containing adapter inducing IFN-β (TRIF) (Yamamoto, Sato et al. 2002; Akira and Sato 2003; Yamamoto, Sato et al. 2003), a

schematic summary of some of the signalling mechanisms is depicted in figure 1.2.2.1. The response that follows will be specific to the type of pattern and thus the microbe and this could mean the induction of immune effector molecules including the natural antimicrobials (Underhill and Ozinsky 2002; Chalifour, Jeannin et al. 2004; Vora, Youdim et al. 2004). MyD88 is an adapter of signal transduction Toll/IL-1R homology (TIR) domain and is recruited following ligation to TLRs (O'Neill 2002). The recruitment of MyD88 results in a signalling cascade which culminates in the nuclear translocation of NF-κB and the activation of the AP-1 transcription factor family (Muzio, Natoli et al. 1998; Kopp and Medzhitov 1999; Cario, Rosenberg et al. 2000; Faure, Equils et al. 2000).

The patterns associated with pathogenic microbes are similar to those associated with commensal organisms. The mechanisms by which the innate immune effectors differentiate between these modalities are hitherto not understood (Kopp and Medzhitov 2003; Chung and Dale 2004). However, it has been demonstrated in the oral cavity that such a distinction is in fact made. Both commensals and pathogens of the mouth have been demonstrated to upregulate the expression of natural antimicrobials; however, this is mediated by different signalling pathways (Chung and Dale 2004; Chung, Hansen et al. 2004).

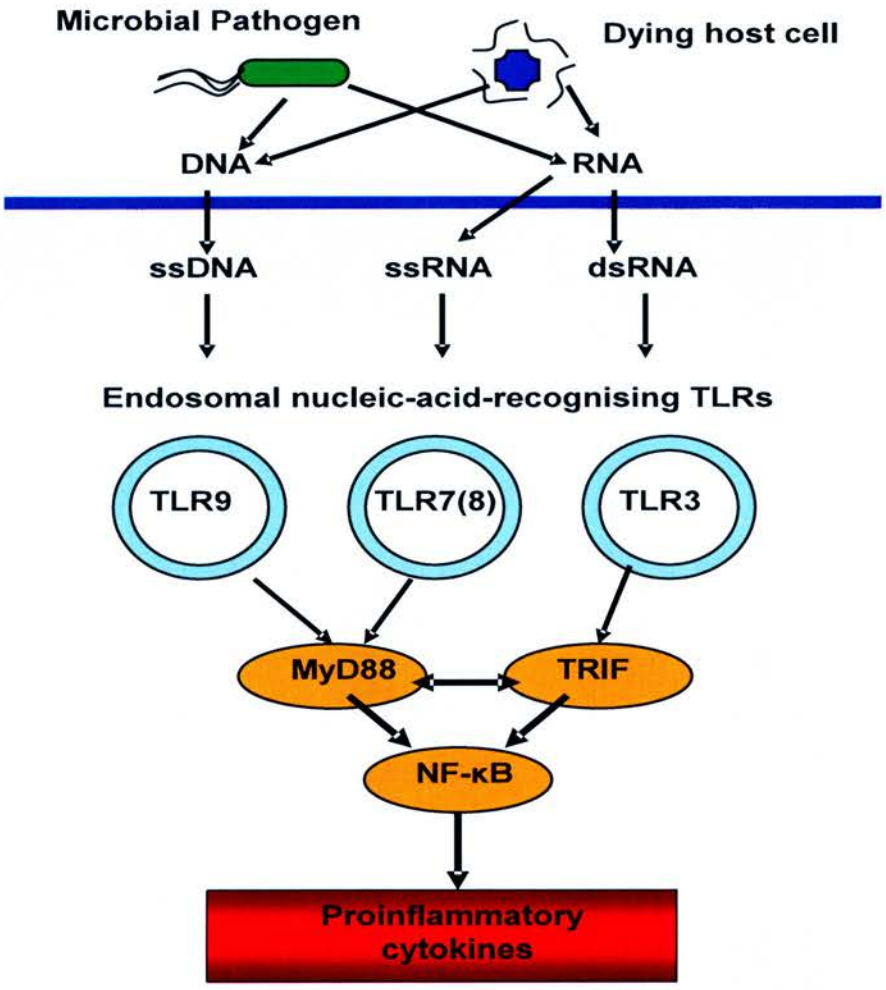


Figure 1.2.2.1 Diagram showing some of the signalling pathways involved in the recognition of pathogens or inflammation. Adapted from Wagner and Bauer (Wagner and Bauer 2006).

1.2.3 Immunity of the female reproductive tract

The role of the immune system as an integrated system has been well documented. The immune system has a pivotal role in the interaction with all other systems of the body, and can no longer be regarded as a 'stand alone' module. Perhaps one of the most complex interactions is that between the immune and reproductive systems. The interplay between the steroid hormones and cytokines has been implicated to be at the heart of these multiple levels of interaction (Wira, Fahey et al. 2005). Although, the interest in these interactions has been relatively recent, the first report of such an interaction is from as early as 1898, where the changes in the thymus after castration were described (Kayisli, Guzeloglu-Kayisli et al. 2004). This has been followed by the evidence that oestrogen treatment enhances extrathymic T – cell maturation (Okuyama, Abo et al. 1992). Further studies on the oestrogen receptor, where both ERα and ERβ knockout mice demonstrated lower levels of thymic cellularity and altered T – cell phenotypes (Staples, Gasiewicz et al. 1999). This was suggestive of interactions between the immune cells and that of the steroid sex hormones (Erlandsson, Ohlsson et al. 2001). The ovarian steroids are responsible for the modulation of a number of immune mediators within the female reproductive tract. The process of menstruation involves the breakdown and repair of tissues and requires the coordination of cellular processes such as apoptosis, angiogenesis, cell proliferation, tissue repair and control of leukocyte populations (Harada, Kaponis et al. 2004). Much of this regulation is mediated via autocrine and paracrine factors from both the non-resident immune cells and resident epithelial and stromal cells of the endometrium (Kayisli, Mahutte et al. 2002).

1.3 Natural antimicrobial peptides

Antimicrobial peptides have been identified in the archea, eubacteria, prokaryotes, protists, plants and invertebrates which suggest that these molecules have been around from early evolution (Hoffmann 1995). The antimicrobial peptides have also been identified within all higher eukaryotes (Boman 1995; Martin, Ganz et al. 1995; Ganz and Lehrer 1999), thus suggesting that they are still an important component of the immune defence. Mammalian natural antimicrobial peptides (NAPs) are expressed mainly within the mucosal epithelia and in the blood, having a pivotal role in the defensive barriers that these structures present. NAPs are small molecules typically around 50 amino acids in length, and have been demonstrated to act directly against bacteria, enveloped viruses and fungi (Aley, Zimmerman et al. 1994; Kragol, Hoffmann et al. 2002). The net charge for the majority of these peptides is positive, which gives rise to the designation as cationic antimicrobial peptides. It has been demonstrated that single amino acid substitutions can greatly affect the function of these peptides (Kragol, Hoffmann et al. 2002). The tertiary structures are diverse with the commonality of their amphipathic nature.

1.3.1 Mechanism of action

Antimicrobial peptides function by means of membrane disruption, causing the target cells to lyse. There have been many models proposed that underpin the exact mechanism by which this occurs. There are 3 models that have been particularly well characterised namely, the carpet, barrel-stave pore and the toroidal pore model (Brogden 2005; Park and Hahm 2005; Duclohier 2006). The carpet model involves

the peptide covering the bacterial membrane surface and an electrostatic reaction results in a detergent like disruption of the membrane. The barrel stave pore mechanism involves the transversing of the target membrane by the peptide, with the hydrophobic surfaces directed towards the lipid membrane and the hydrophilic surfaces facing inwards. The resultant pore allows for membrane leakage of water and electrolytes (Oren and Shai 1998). The toroidal pore model describes a similar mechanism however, suggests that the peptides form aggregates that cause the lipid monolayers to bend resulting with the lipid head groups facing the water core (Allende, Simon et al. 2005).

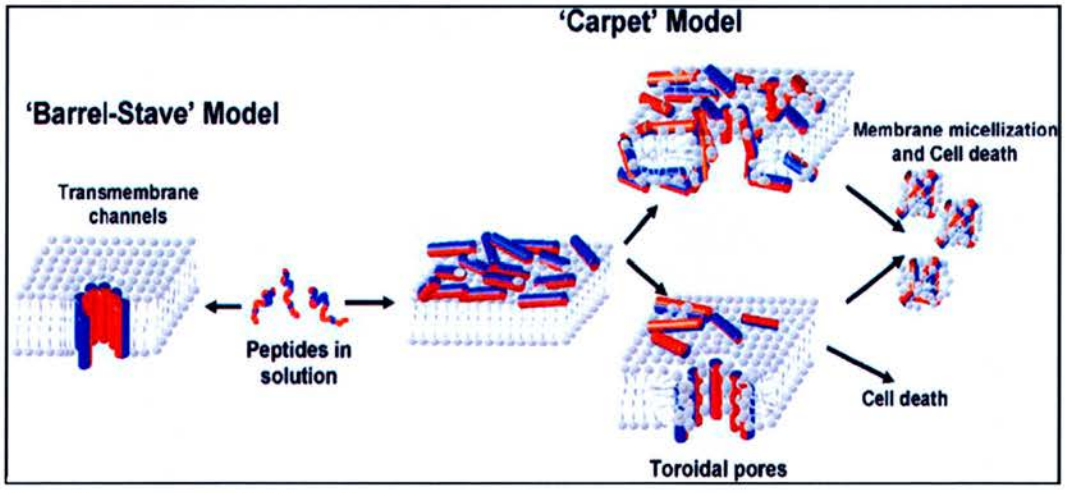


Figure 1.3.1.1 Diagram taken from Duclohier (Duclohier 2006), demonstrating the 3 proposed models for the mechanisms employed by antimicrobial peptides in interrupting microbial cell membranes.

The natural antimicrobial peptides target the membranes of microbes, these membranes are sufficiently finely tuned that modification or adaptation is difficult to achieve. Thus, bacterial resistance to natural antimicrobial peptides has rarely been

observed (Hancock and Diamond 2000). The differences between that of the bacterial and mammalian membranes, means that the peptides preferentially attack that of the bacteria. This is due to the fact that the cationic peptides are attracted to the negatively charged membranes as opposed to the neutrally charged mammalian membranes. Bacterial membranes have a higher proportion of acidic phospholipids, whilst eukaryotic membranes are made up of cholesterol and zwitterionic phospholipids on the outer membrane, with the acidic phospholipids located on the inside (Zasloff 2002). The lysis of the target cell is a well characterised mechanism of action, however, other methods have been described (Brogden 2005). For example antimicrobials which are proline-rich target intracellular mechanisms, such as protein synthesis (Boman, Agerberth et al. 1993; Gennaro, Zanetti et al. 2002). Whilst, the antifungal histatins, target the function of the mitochondria affecting ATP efflux (Kavanagh and Dowd 2004). Many natural antimicrobials have additional non-antimicrobial functions (Hancock and Diamond 2000). This is discussed further in section 1.3.2 and section 1.4.3.

1.3.2 Other roles for antimicrobial peptides

It has been increasingly documented that asides from the role in the killing or inhibition of microbials, the antimicrobial peptides have other functions within the immune system (Hancock and Diamond 2000). Antimicrobial peptides have been shown to be chemotactic and thus, serve to attract other immune effectors such as leukocytes (Agerberth, Charo et al. 2000), monocytes (Territo, Ganz et al. 1989) and T-cells, functioning within both the innate and adaptive immune system (Chertov, Michiel et al. 1996; Agerberth, Charo et al. 2000). The use of has also demonstrated

that these peptides have an immunomodulatory role. They can mediate the upregulation of chemokines in epithelial cells and macrophages, and modulate the differentiation of dendritic cells, thereby mediating their endocytic capacity (Davidson, Currie et al. 2004; Bowdish, Davidson et al. 2005; Tjabringa, Vos et al. 2005). The role of these peptides in the modulation of the immune response is so crucial; it is raising the question, of whether the antimicrobial effects are indeed the primary function of these cells (Bowdish, Davidson et al. 2005; Bowdish, Davidson et al. 2006). They are also involved in the neutralisation of LPS, increasing phagocytosis, induction of mast cell degranulation and in the mediation of the complement system (Larrick, Hirata et al. 1994; Larrick, Hirata et al. 1995; Sawa, Kurahashi et al. 1998; Rosenfeld, Papo et al. 2006). A further multifunctional role has been attributed to these molecules with an involvement in the process of angiogenesis and re-epithelisation of wounds (Li, Post et al. 2000; Heilborn, Nilsson et al. 2003; Galkowska, Olszewski et al. 2005).

1.3.3 Mammalian natural antimicrobial peptides

There are two major families of antimicrobial peptides expressed in mammals, the Defensins and the cathelicidins, each with their own characteristics. The Defensins are characterised by the presence of 3 disulphide bonds (Lehrer, Lichtenstein et al. 1993), whilst the cathelicidin family are recognised by the evolutionarily conserved prepro region (Zanetti, Gennaro et al. 1995; Tomasinsig and Zanetti 2005; Zanetti 2005).

The Defensins are further subdivided into distinct groupings based upon differences within their respective structures. The 3 groups are designated α- β- and θ-Defensins. The α- and β-defensins have the characteristic defensin structure, which consists of amphipathic β-sheet rich region, but, differ in their primary structure and positioning of the disulphide bonds (Ganz 2003). The α-defensins are expressed mainly by neutrophils and by the paneth cells of the gut. They are expressed as prepro-proteins requiring subsequent processing into mature peptides, which are typically ~ 30 amino acids in length. Whilst the β-defensins are expressed mainly by the epithelial cells, as pre-proteins and are spliced to produce mature peptides of ~ 35 – 45 residues in length (Ganz 2003).

The θ-defensins were identified in the leukocytes of rhesus macaques and are circular in shape, formed via the ligation of two short peptides (Tang, Yuan et al. 1999). These molecules have been demonstrated to act against both bacteria and fungi. It was further demonstrated that the circular structure of this 18 residue peptide is important for function as linear analogues were less active (Tran, Tran et al. 2002).

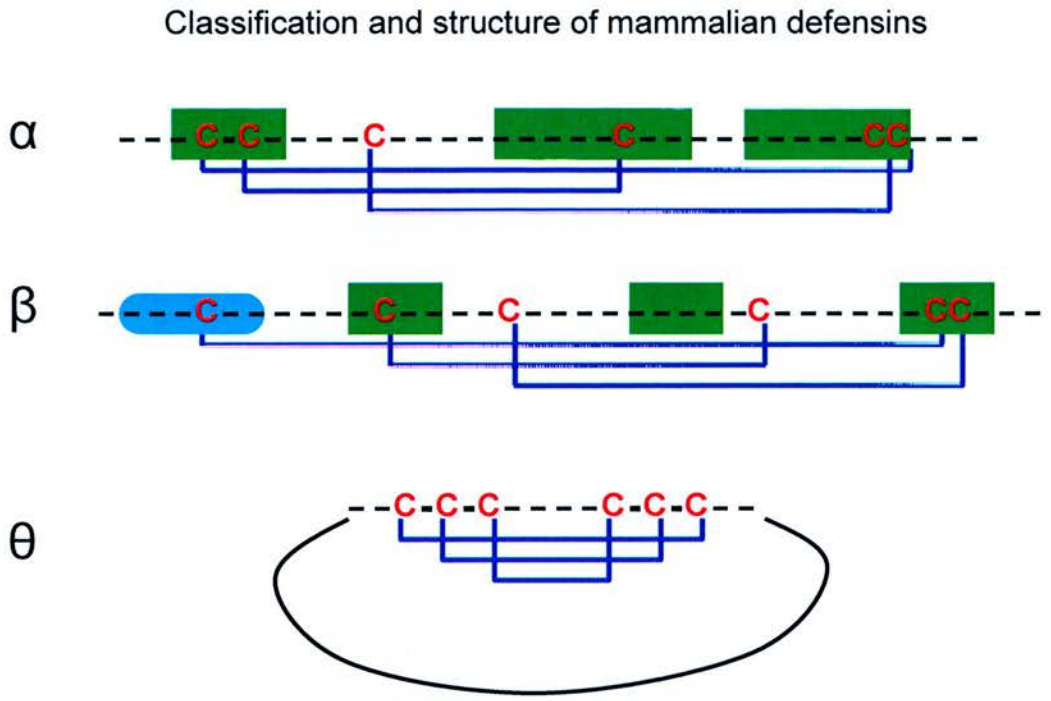


Figure 1.3.3.1 An illustration of the structure for each class of the mammalian defensins (α, β and θ). The conserved cysteines (C) and consensus sequence for each defensin are illustrated by dashed lines, with each dash representing a residue. The green highlighted areas demonstrate the position of β-sheets, whilst the blue shows the α-helice region. The disulphide bonds are illustrated by the blue lines. Adapted from Yang et al. (Yang, Biragyn et al. 2002).

1.3.4 Human natural antimicrobial peptides

In humans the α-defensin family consists of 6 members; HNP1-4, the human neutrophils peptides, which are expressed by neutrophils (Lehrer, Lichtenstein et al. 1993) and HD5-6, human Defensins 5 and 6, expressed by the paneth cells of the

small intestine (Jones and Bevins 1992; Jones and Bevins 1993). HNP1-3 are stored within the neutrophilic granules as mature peptides that become active when the granules are fused with phago-lysozomes (Ganz 2003). Whilst HD5 has been demonstrated to be stored within paneth cells as a pro-peptide, and proteolytically cleaved by paneth cell trypsin (Jones and Bevins 1993).

There are at least 28 different human β-defensins that have been identified within the genome (Schutte, Mitros et al. 2002; Semple, Gautier et al. 2006) of which four have been characterised, hBD1-4 (Bensch, Raida et al. 1995; Harder, Bartels et al. 1997; Garcia, Jaumann et al. 2001; Garcia, Krause et al. 2001; Harder, Bartels et al. 2001). They are mainly expressed by the epithelia and will be discussed in more detail as they are investigated in this thesis.

There have been 6 θ-defensins identified within humans, however the presence of premature stop codons, designates these as pseudogenes (Nguyen, Cole et al. 2003). However, their potential in therapeutics has been suggested by the existence of an mRNA transcript for one of these genes within bone marrow. A synthetic replicate of this potential protein, retrocyclin, was shown to protect cells from infection with HIV (Cole, Hong et al. 2002; Munk, Wei et al. 2003).

There has been only one cathelicidin identified in humans, LL-37, the name is derived from the structure – LL from the N-terminal peptides (Leucine, Leucine), and the mature peptide is 37 residues in length (Gudmundsson, Agerberth et al.

1996). However, the peptide exists in a proform, and is often referred to as hCAP18 (Larrick, Hirata et al. 1995).

1.4 Defensins

The mammalian defensins are variable in size and structure and characterised by the position of 6 invariant cysteines in the formation of triple stranded β-sheets. The division of these peptides into α and β is based on differences at both the peptide and gene level. The tertiary structures of both the α and β defensins are almost superimposable (Zimmermann, Legault et al. 1995), the differences are attributable to the position of the cysteine residues and their disulphide bonds (Huttner and Bevins 1999; Raj and Dentino 2002).

1.4.1 α-defensins

Human α-defensins are expressed in neutrophils, granulocytes (HNP1-4), in the paneth cells of the small intestine (HD5 and 6) (Lehrer, Lichtenstein et al. 1993; Diamond and Bevins 1998; Folkvord, McCarter et al. 2006) and in the female reproductive tract. HD5 has been found throughout the reproductive tract including the endometrium, Fallopian tubes, vagina, ectocervix, endocervix and chorion (Svinarich, Wolf et al. 1997; Quayle, Porter et al. 1998). HNP1-3 have been identified in human vernix caseosa and amniotic fluid, which may reflect a role in neonate immunity (Yoshio, Tollin et al. 2003). The endometrial expression of HD5 was found to be maximal during the early secretory phase of the menstrual cycle (Quayle, Porter et al. 1998). The α-defensins are expressed in a pre-propeptide form

and undergo post-translational modification, which can occur within cytoplasmic granules of neutrophils or in lumen of the intestine. It was demonstrated that in the murine intestine there is a co-localisation of MMP7 (matrilysin) in the paneth cells. In vitro studies subsequently demonstrated the ability of the metalloprotease to cleave the pro-segment of these peptides into their mature and active form (Wilson, Ouellette et al. 1999). In the human paneth cells, α-defensins are stored as pro-peptides which are proteolytically cleaved by trypsin in the lumen of the gut (Ghosh, Porter et al. 2002; Weeks, Tanabe et al. 2006).

1.4.2 β-defensins

There are currently 4 well characterised human β-defensins (hBD1-4) and are localised to the epithelial cells, monocytes and alveolar macrophages. Human β-defensin 1 and 2 were first described in the skin, saliva, lung and plasma (Bensch, Raida et al. 1995; Zhao, Wang et al. 1996; Harder, Bartels et al. 1997; Bals, Wang et al. 1998). hBD1 and 2 have also been isolated from the majority of the female reproductive tract, including the mucosa of the vagina and cervix, ectocervix, endocervix, uterus and the fallopian tubes (Valore, Park et al. 1998). It has also been demonstrated that there is differential expression of hBD1-4 in the endometrium in response to the menstrual cycle. Human β-defensin 1 shown to be expressed throughout the cycle, hBD2 to be maximal during menses, hBD3 maximally expressed during early to mid secretory phase and hBD4 maximally expressed during the proliferative phase of the cycle (King, Critchley et al. 2003; King, Fleming et al. 2003). HBD1-3 have also been identified in the fetal membrane and placenta (King,

Paltoo et al. 2007). Human β-defensin-3 has also been isolated from the heart, skeletal muscle, placenta, skin, testis, oesophagus, gingival keratinocytes and trachea (Garcia, Jaumann et al. 2001; Jia, Schutte et al. 2001). Human β-defensin 4 is the least well understood but has been identified in the testis, uterus, lung, kidney and thyroid gland in response to infection (Garcia, Krause et al. 2001). There have been a growing number of defensin molecules that have been identified via a genomics based approach (Jia, Schutte et al. 2001; Scheetz, Bartlett et al. 2002; Schutte, Mitros et al. 2002) and presenting with many opportunities for the research into the function of these immune effectors (Semple, Gautier et al. 2006; Semple, Taylor et al. 2006).

The genes for the β-defensins have all been localised to gene clusters on chromosomes 6, 8 and 20 (Schutte, Mitros et al. 2002). Important consensus sites have also been identified. The 5'-flanking region of hBD1 has the consensus sites for nuclear factor interleukin 6 (NF-IL-6) and IFNγ (Raj and Dentino 2002); hBD2 has NF-κB, NF-IL-6, STAT and AP-1 (Tsutsumi-Ishii and Nagaoka 2002); hBD3 has NF-IL-6, STAT and AP1 consensus sites (Jia, Schutte et al. 2001). These consensus sites are inflammatory mediated sites that are common to immune effectors; their presence suggests that hBD expression is mediated by inflammatory mediators such as interleukins.

The hBDs are mainly expressed by the epithelia in a constitutive manner, and in some cases are upregulated in response to infection (Zhao, Wang et al. 1996; Krisanaprakornkit, Weinberg et al. 1998; Liu, Wang et al. 1998; Krisanaprakornkit, Kimball et al. 2000; Krisanaprakornkit, Kimball et al. 2002; Liu, Destoumieux et al.

2002). The process whereby the defensins are upregulated in response to infection or inflammatory stimuli has been demonstrated using *in vitro* studies. The use of LPS (G-ve mimic), and inflammatory cytokines such as IL-1 and TNFα to stimulate inflammatory events have been deployed in systems such as the lung, gut and gingival linings (Diamond, Russell et al. 1996; O'Neil, Porter et al. 1999; Krisanaprakornkit, Kimball et al. 2000; O'Neil, Cole et al. 2000; Harder, Bartels et al. 2001). The use of inflammatory mimics have also been utilised for the investigation of defensin expression in the female reproductive tract (King, Fleming et al. 2002) and this was also demonstrated to be effective in obtaining an inflammatory response with upregulation of antimicrobial expression.

The defensins that have been identified and characterised have been shown to be microbicidal against a range of bacteria, fungi and viruses (Ganz, Selsted et al. 1985; Miyasaki, Bodeau et al. 1990; Ganz, Oren et al. 1992; Lehrer, Lichtenstein et al. 1993; Harwig, Ganz et al. 1994; Porter, van Dam et al. 1997; Huang, Zhang et al. 2002; Lee, Andalibi et al. 2004).

The defensins have been reported to have different specificities or 'specialities', with some being more potent against G+ve or G-ve bacteria, or against fungal agents or viruses (Ganz, Selsted et al. 1985; Risso 2000). This effect may also be governed by their respective locale, in that they may not respond to an organism that they ordinarily would not expect to be exposed (Raj and Dentino 2002). They are also affected by pH for example it has been demonstrated that sodium chloride is inhibitory to hBD activity (Goldman, Anderson et al. 1997; Bals, Wang et al. 1998;

Garcia, Krause et al. 2001). It has been reported that hBD3 is resistant to inhibition in the presense of salt, which is significant with regard to cystic fibrosis (CF) (Harder, Bartels et al. 2001), as it has been suggested that the higher physiologic concentrations of salt in these patients is inhibitory to their innate defensins (Guggino 1999).

1.4.3 Natural antimicrobial peptides as immune effectors

Antimicrobial peptides have been demonstrated to have roles within the immune system distinct from their microbicidal function. They act as chemoattractants for cells from both the innate and adaptive immune systems (Agerberth, Charo et al. 2000). This suggests that the peptides are able to function at the interface between the innate and adaptive response. The immunomodulatory role of these peptides has been demonstrated in a variety of primary and cultured cell lines. Davidson et al, reported the role of LL-37 in the modulation of dendritic cell differentiation and enhancing their endocytic capacity (Davidson, Currie et al. 2004). Further evidence from the same laboratory has described the regulation of cytokines and a role in the process of apoptosis by LL-37 (Bowdish, Davidson et al. 2005; Barlow, Li et al. 2006). The α-defensins have been shown to exhibit a role in the processing of mature IL-1β and thereby enhancing the production of this cytokine. The β-defensins have been shown to both act directly as and upregulate other chemokines, attracting immune effectors to source of infection or injury (Perregaux, Bhavsar et al. 2002; Bowdish, Davidson et al. 2006). The β-defensins have also been shown to upregulate the expression of inflammatory cytokines in peripheral blood mononuclear cells (Boniotto, Jordan et al. 2006).

1.5 Natural antimicrobial proteins

There are a number of proteins that have proved to exhibit antimicrobial activities. The past two decades have given rise to a great deal of publication and research into the function of innate antimicrobial proteins. However, this potential was first realised as early as 1922, when Alexander Fleming reported the "remarkable bacteriolytic element" (Fleming 1922) and "a powerful antibacterial ferment which had a most extraordinary lytic effect on some bacteria" in his description of lysozyme during his Nobel Laureate Lecture in 1945 (Fleming 1945). An increasing number of proteins exhibiting antimicrobial properties have been described or isolated from a wide variety of sources from plants, insects and mammals. However, the antimicrobial proteins that are of interest to this thesis are discussed further within this review and within the relevant data chapters.

1.5.1 Granulysin

Granulysin was first described in 1997, and is mainly produced by Natural Killer (NK) cells and CD8 T-cells (Pena, Hanson et al. 1997; Pena and Krensky 1997). The protein is 9 kDa and is a member of the saposin-like family and is colocalised with granzyme and perforin within cytolytic granules. Granulysin has been shown to have microbicidal effects upon both Gram +ve and Gram -ve species of bacteria, fungi and parasites (Stenger, Rosat et al. 1999; Walch, Eppler et al. 2005). It has also been reported that Granluysin along with perforin to have the abiltity to kill cancerous tumours (Kishi, Takamori et al. 2002). Granulysin also has a role with the

modulation of the immune response, and has been shown to be chemotactic for monocytes, NK, dendritic and T cells (Deng, Chen et al. 2005). Granulysin is also thought to have a role within the endometrium during the menstrual cycle with maximal expression being observed during the late secretory phase and is likely to be expressed by the infiltrating uterine NK (uNK) cells (King, Critchley et al. 2003). The levels of granulysin have also been shown to be elevated during pre-eclampsia and may serve as a useful diagnostic marker for the Th1/Th2 balance and thus, cell mediated immunity (Sakai, Ogawa et al. 2004). Further diagnostic application has been described with the presence of granulysin mRNA detected in urinary sediment, providing an indication of a renal allograft rejection (Kotsch, Mashreghi et al. 2004).

1.5.2 Secretory Leukocyte Protease Inhibitor (SLPI)

SLPI was originally identified for being a serine protease inhibitor and was found in the secretions obtained from mucosal surfaces. SLPI is 11.7 kDa, consisting of 107 amino acids including 16 cysteine residues (Seemuller, Arnhold et al. 1986) and consists of two WAP (whey acid protein)/four disulphide core domains. SLPI has been demonstrated to inhibit a variety of different proteases (Hiemstra 2002) and to exhibit antimicrobial properties. However, the antimicrobial action of this protein is thought to be independent of its anti-protease function. SLPI has been demonstrated to be microbicidal against both G+ve and G-ve bacteria (Hiemstra, Maassen et al. 1996), fungi (Tomee, Hiemstra et al. 1997) and viruses, most notably HIV-1 (McNeely, Shugars et al. 1997; Wahl, McNeely et al. 1997; Shugars 1999; Shugars,

Alexander et al. 1999). SLPI has been reported to be expressed by both neutrophils (Bohm, Aigner et al. 1992) and epithelial cells (Abe, Kobayashi et al. 1991).

The anti-protease action of SLPI was originally described within the lung as being an inhibitor of neutrophil elastase and has also been shown to exert this action against cathepsin G, trypsin and chymotrypsin (Thompson and Ohlsson 1986). This anti-protease activity serves to prevent damage to normal tissues during an inflammatory response. This anti-inflammatory role has been further defined by the inhibition of histamine (Dietze, Sommerhoff et al. 1990; He, Xie et al. 2004), and the prevention of NF-κB activation (Lentsch, Jordan et al. 1999; Taggart, Cryan et al. 2005). Thus, preventing further inflammation and damage (Ward and Lentsch 2002). It has been reported to have an inhibitory effect upon the production of MMPs by monocytes via the indirect action on PGE_2 (Zhang, DeWitt et al. 1997). It is also thought that SLPI has a role in the facilitation of wound repair, as SLPI knockout mice demonstrated an impaired ability in cutaneous wound repair along with increased levels of elastase and accompanying inflammation (Ashcroft, Lei et al. 2000; Angelov, Moutsopoulos et al. 2004).

SLPI has been isolated from a variety of mucosal secretions and has been identified amongst other immune effectors to have a site specific profile of expression (Tjabringa, Vos et al. 2005). It is suggested that this site specific profile may determine the role of SLPI as an immune effector across different mucosal environs. SLPI has been shown to have antibacterial activity against a spectrum of different organisms. This activity has been demonstrated against *Escherichia coli* and

Staphylococcus aureus (Hiemstra, Maassen et al. 1996); *P. aeruginosa* and *S. epidermis* from skin infections (Wiedow, Harder et al. 1998); and pathogens of the gastrointestinal tract, *Salmonella Typhimurium* (Si-Tahar, Merlin et al. 2000), and the downregulation of SLPI has been implicated in *Helicobacter pylori* mediated gastritis (Wex, Ye et al. 2006). In the last decade the anti-viral activity of SLPI against HIV-1 has been well documented, *in vitro* SLPI isolated from saliva was shown to be inhibitory (McNeely, Dealy et al. 1995) and there are elevated levels of SLPI in response to infection with HIV-1 (Skott, Lucht et al. 2002). It has also been reported that lower endogenous levels of SLPI within vaginal fluids resulted in a higher level of HIV-1, and thus, a greater level of maternal transfer (Pillay, Coutsoudis et al. 2001).

The gene encoding SLPI has been located on chromosome 20q12 (Kikuchi, Abe et al. 1998). The promoter has been reported to have sites for the transcription factors AP-1 and AP-2 (Abe, Kobayashi et al. 1991). The protein is composed of two homologous domains and it has been shown that they have distinct roles. The C terminal region is responsible for the anti-protease properties described (Eisenberg, Hale et al. 1990; Kramps, van Twisk et al. 1990), whilst the N terminal region has antimicrobial actions (Hiemstra, Maassen et al. 1996).

However, the precise mechanism of this antimicrobial function is not yet understood. SLPI is cationic (isoelectric point =10.5) (Schnebli 1991), so it may be that this enables a mechanism similar to that described for the defensins (Zelvyte, Wallmark et al. 2004). SLPI is also similar to the defensins in respect of the molecules salt

sensitivity, the microbicidal actions of SLPI are reduced in higher salt conditions (Hiemstra, Maassen et al. 1996).

1.5.3 Elafin

Elafin is also known as SKALP (skin derived anti-leukoprotease) and ESI (elastase specific inhibitor). The 9.9 kDa protein was first isolated from skin samples obtained from patients with psoriasis and in the lung, where it was described as an elastase inhibitor (Schalkwijk, Chang et al. 1990; Sallenave and Ryle 1991; Wiedow, Young et al. 1993). Elafin has two functional domains, consisting of a C terminus which exhibits similarities to the WAP/four-disulphide core domain found in SLPI. The N terminus is a region rich in Gln and Lys residue repeats and is known as the cementoin domain and acts as a substrate for transglutaminase (Nara, Ito et al. 1994; Tremblay, Vachon et al. 2002; Guyot, Zani et al. 2005). This domain has been identified as a feature of the trappin family, of which elafin is the second member – trappin-2. Trappin is an acronym for TRansglutaminase substrate and wAP domain containing ProteIN, and is named due to the observation that the proteins are 'trapped' in tissues via covalent crosslinking (Schalkwijk, Wiedow et al. 1999). The term trappin-2 (pre-elafin) refers to the whole molecule, whilst the term elafin is more accurately used to describe the 6 kDa molecule which results after cleavage (Zeeuwen, Hendriks et al. 1997; Schalkwijk, Wiedow et al. 1999). This truncated molecule consists of the C-terminal domain which lacks the cementoin region. It has been shown that when elafin lacks the cementoin the molecule is less effective as an antiprotease in an *in vivo* model of elastase-induced injury to the lung (Tremblay,

Vachon et al. 2002). Thus, it is believed that it is intact elafin (trappin-2), which is the most effective as an antiprotease in the protection of tissues from elastase mediated inflammation and damage.

Elafin was first characterised in psoriatic skin and is characterised with the high level of neutrophil influx that is associated with this inflammatory condition (Alkemade, Molhuizen et al. 1994; Nonomura, Yamanishi et al. 1994). The squamous epithelium of the skin serves as an important barrier and as such is continuously exposed to inflammatory stimuli and it has been shown that elafin expression is constitutive within this region (Wiedow, Schroder et al. 1990; Pfundt, van Ruissen et al. 1996). The expression of elafin in the lungs has also been well documented, intitially detected in bronchial secretions (Sallenave, Marsden et al. 1992; Sallenave and Silva 1993). Further studies identified that elafin was expressed by the bronchial epithelial cells (Sallenave, Shulmann et al. 1994), alveolar epithelial cells (Sallenave, Silva et al. 1993) and alveolar macrophages (Mihaila and Tremblay 2001). Elafin expression has also been identified in the epithelia of the large intestine (Suzuki, Furukawa et al. 2000), and recently a downregulation of both elafin and SLPI has been associated with the inflammatory bowel condition Crohn's disease (Schmid, Fellermann et al. 2007). Epithelial expression of elafin has also been described in the endometrium (King, Critchley et al. 2003), amnion (Tromp, Kuivaniemi et al. 2004; King, Paltoo et al. 2007) and has also been observed in the neutrophil population during menstruation (King, Critchley et al. 2003) (section 1.6).

The use of cell lines and *in vitro* models have demonstrated the inducibility of elafin in the presence of the inflammatory cytokines TNFα and IL-1 in the lung (Sallenave, Shulmann et al. 1994), keratinocytes (Tanaka, Fujioka et al. 2000) endometrium (King, Fleming et al. 2002) and mammary cells (Zhang, Magit et al. 1997). The expression of elafin upon treatment with these pro-inflammatory cytokines may be mediated via the c-jun, p38 mitogen activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways (Pfundt, Wingens et al. 2000; Bingle, Tetley et al. 2001; Pfundt, van Vlijmen-Willems et al. 2001).

1.6 Natural antimicrobials in the female reproductive tract

The female reproductive tract consists of three distinct areas: the lower reproductive tract which includes the vagina and ectocervix; the upper reproductive tract which consists of the endocervix, which acts as an interface between the upper and lower areas, the endometrium and the Fallopian tubes (Quayle 2002). The lower reproductive tract is non-sterile, whilst the upper reproductive tract is considered to be sterile in healthy women. The sterility of the ectocervix is variable in relation to the phase of the menstrual cycle (Quayle 2002). The lower reproductive tract is described as non-sterile due to the presence of a number of commensal organisms. The presence of commensals such as *Lactobacillus* spp are thought to function as part of the immune defense of the lower reproductive tract which is potentially exposed to a number of pathogens and sexually transmitted diseases (STDs). Despite the potential exposure and increasing prevalence of STDs (Cherpes, Meyn et al. 2003), the innate immune system of the female reproductive tract is poorly

understood. However, recently it has been shown that the role of natural antimicrobials is a major component in the maintenance of an infection free reproductive tract, whilst tolerating the commensal population (Yedery and Reddy 2005). The natural antimicrobials have been demonstrated to be differentially expressed throughtout the reproductive tract and this is further variable in response to changes associated with the ovarian cycle (King, Critchley et al. 2003). However, it is clear that there is broad coverage and this may be suggestive of the distinct roles, mechanisms and interactions of the antimicrobials within the reproductive tract. A summary of the major literature reports of natural antimicrobial expression within the female reproductive tract is presented in figure 1.6.1.

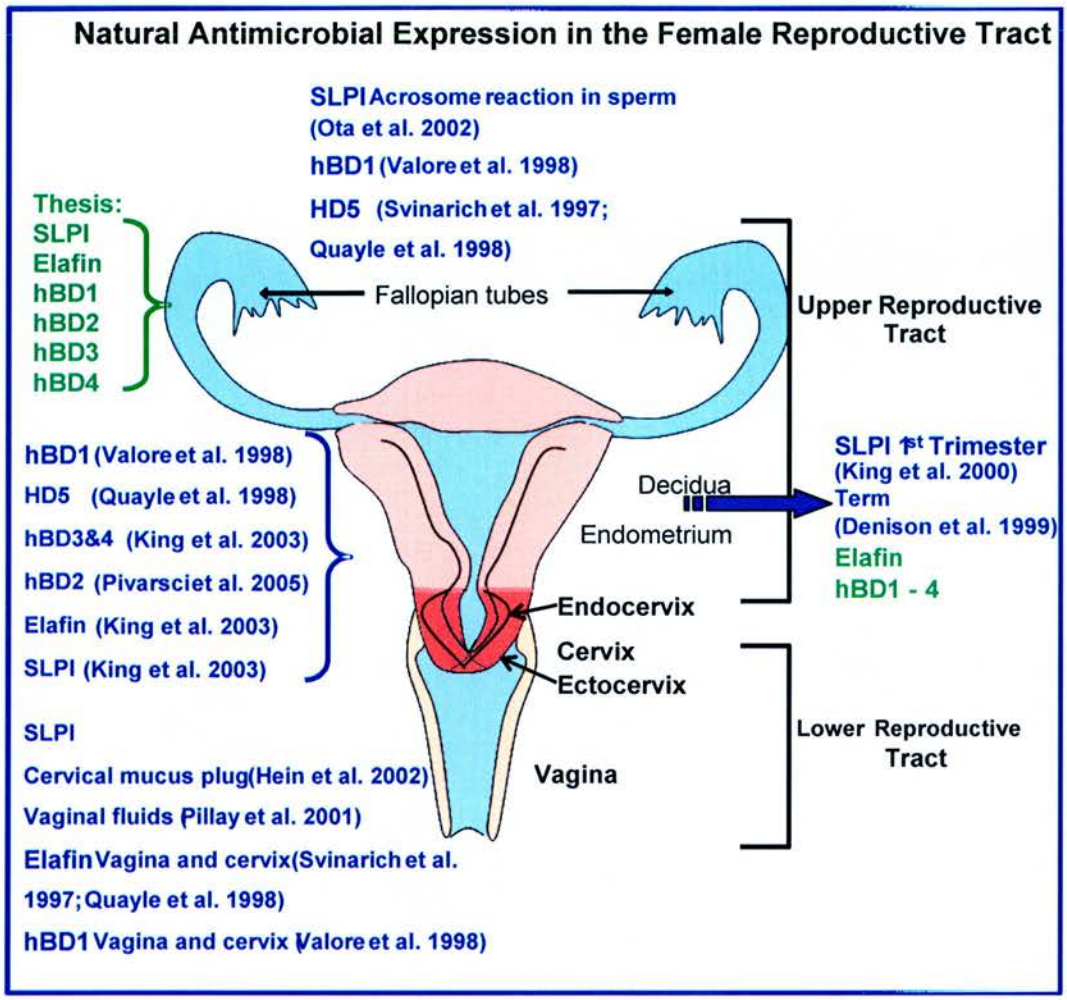


Figure 1.6.1 A summary of natural antimicrobial expression in the female reproductive tract, those reported in the literature (blue) and those described in the current thesis and not yet published (green).

1.7 Inflammatory mediators within the female reproductive tract

The process of menstruation and implantation have been likened to inflammatory events (Finn 1986; Salamonsen and Woolley 1999; Kelly, King et al. 2001). A number of inflammatory mediators have been described as having a role within the endometrium. These include cytokines, chemokines and prostaglandins, with a more recent role being elucidated for natural antimicrobial peptides.

1.7.1 Cytokines and growth factors

Cytokines are soluble polypeptides and are primarily involved with the immune response with both autocrine and paracrine interactions. They are synthesised and secreted by immune effector cells (Janeway, Travers et al. 2001). Cytokines are also expressed by the epithelial, stromal and endothelial components of the endometrium. They are involved with both the induction and resolution of immune inflammation. Thus, some are described as being pro-inflammatory or anti-inflammatory. Cells expressing cytokines can be classified according to their cytokine production as either Th-1 or Th-2 (Mossmann, Bamberger et al. 1986). The Th-1 cytokines include IFN-γ, IL-2, IL-12 and TNFα, whilst examples of Th-2 cytokines include IL-4, IL-10 and IL-13.

Cytokines have a role to play within the endometrium in both the pregnant and non-pregnant state (Tabibzadeh 1991; Kelly, King et al. 2001). Cytokines also have roles in neuroendocrine events via their interactions with pituitary and hypothalamic hormones (Druker, Liberman et al. 2006).

1.7.1.1 Interleukin – 1 (IL-1)

IL-1 is a multi-functional cytokine and exists in two forms, IL-1α and IL-1β. There is very little sequence homology (27%) between the two forms; however, they elicit the same actions and utilise the same receptor, IL-1 type-1 (IL-1RI) (Dinarello 1991). A second receptor exists, IL-1 type-2 (IL-1RII), however, to which no function has yet been attributed (Sims and Smith 2003). There is a receptor

antagonist (IL-1ra), which is secreted but, has also been located within decidual glands, in isolated cells of the chorionic villus, intervillous space and within maternal decidua (Simon and Polan 1994). In the human endometrium both IL-1α and IL-1β are expressed by the epithelial and stromal components (Tabibzadeh and Sun 1992; Simon, Piquette et al. 1993). The levels of IL-1 are variable across the menstrual cycle and are at their highest during the secretory phase, post ovulation (Rodgers, Matrisian et al. 1994). Interleukin-1 has been connected with the process of implantation and is expressed in both trophoblast and decidual cells along with the receptor (Simon and Polan 1994; Simon, Pellicer et al. 1995). Endometrial stromal cells (ESCs) elicit an immune response upon treatment with IL-1α, and have been demonstrated to phagocytose latex beads and *E. coli* (Ruiz, Montes et al. 1997). This cytokine has also been shown to stimulate the production of MMP-1, which is involved in the initiation of menstruation (Singer, Marbaix et al. 1997). Interleukin-1β increases the levels of PGE_2 in decidual and endometrial ESCs (Cole, Seki et al. 1995; Cole, Seki et al. 1995; Ishihara, Matsuoka et al. 1995). Interleukin-1α stimulates PGE_2 and $PGF_{2\alpha}$ production in ESCs (Kawano, Nakamura et al. 2001). The actions of both IL-1 types is suggestive of a role in the regulation of menstruation, via the stimulation of prostaglandins which are thought to be involved in the vascular changes associated with the degradation of the endometrium (Baird, Cameron et al. 1996; Gerard, Caillaud et al. 2004).

Interleukin-1 secretion can be induced by a number of factors including other cytokines such as TNFα and IFN-γ and can also be upregulated in response to bacterial products (Gerard, Caillaud et al. 2004). There are also a number of

inhibitors of IL-1 secretion, including PGE_2, IL-6 and glucocorticoids (Tabibzadeh, Kaffka et al. 1990). Interleukin-1 has been associated with a number of important functions, activating T-helper cells and NK cells; stimulation of immunoglobulin production; fibroblast proliferation and chemotaxis for neutrophils (Gerard, Caillaud et al. 2004).

1.7.1.2 IL-1 Receptor

The IL-1 receptors 1 and 2 have extracellular domains which identifies them as belonging to the immunoglobulin superfamily. It has been shown that after IL-1 binds to the type 1 receptor, an accessory protein docks thereby bringing about high affinity binding (Sims and Smith 2003). The type 1 receptor has a structure comprising of a single transmembrane segment and cytoplasmic domain. In the type 2 receptor this signalling cytoplasmic domain is absent (Gerard, Caillaud et al. 2004).

Once binding has occurred, a cascade of biochemical events ensue. This involves the sequential phosphorylation and/or dephosphorylation of a number of kinases. This results in the nuclear translocation of transcription factors and activation of proteins, resulting in the translation of RNA. Amongst the proteins activated is the adapter protein MyD88, IL-1 Receptor Associated Kinases (IRAK), and TNF Receptor-Associated Factor-6 (TRAF-6). However, most of the effects of IL-1 are following the translocation of NFκB (nuclear factor-kappa B) and activating protein-1 (AP-1) (Daun and Fenton 2000). The binding sites for NFκB and AP-1 are present in the promoter regions of many of the IL-1 inducible genes including PGE_2, IL-6 and IL-8.

NFκB is an important transcription factor that is responsible for the activation of a number of genes involved with both the innate and adaptive immune response.

1.7.1.3 Tumour Necrosis Factor α (TNFα)

TNFα is secreted by a wide variety of cells including macrophages, monocytes, neutrophils, T lymphocytes, NK cells, fibroblasts and granulosa cells. TNFα mediates its action through binding to cell bound receptors, p55 and p75 which belong to the TNF-R gene family (Bazzoni and Beutler 1996). These receptors are structurally similar but, exhibit independent functions and are also found in the soluble form, in the circulation. The role of these soluble receptors is thought to be as antagonists to the membrane forms, whilst it is also possible that the half-life of circulating TNFα is increased in the presence of soluble receptors (Mohler, Torrance et al. 1993).

In the endometrium TNFα has been shown to be expressed within the glandular epithelium (Tabibzadeh 1991; Hunt, Chen et al. 1992; Tabibzadeh, Kong et al. 1994; Tabibzadeh, Kong et al. 1995; Tabibzadeh, Zupi et al. 1995). The expression of TNFα during the menstrual phase and the defined biological activity of the cytokine suggests that it may have a role in the process of menstruation (Tabibzadeh, Zupi et al. 1995; Tabibzadeh 1996; von Wolff, Thaler et al. 2000). Hunt et al (Hunt, Chen et al. 1992), have shown TNFα mRNA to be low in the proliferative phase and is increased during the secretory phase and this was later confirmed by others (Philippeaux and Piguet 1993). However, in the late secretory phase TNFα declines

within the glandular epithelium, whilst the stromal cells show strong expression (Hunt, Chen et al. 1992).

The expression of TNFα can result from a number of inducible factors, such as bacterial products, tumours and other cytokines such as IFN-γ. However, there is also expression of TNFα in normal tissue which is unlikely to be as a result of bacterial products or other infection stimuli. There are also a wide range of factors which have an inhibitory effect upon TNFα. Inhibitors include prostaglandins, glucocorticoids and anti-inflammatory cytokines, such as IL-4 and IL-10. The role of TNFα within the endometrium is undetermined, but, may have a role similar to that of IL-1, acting as part of the immune defence and the processes of menstruation and implantation. The known biological actions of TNFα are wide ranging and are pro-inflammatory (Svensson, Mardh et al. 1985), cytotoxic and chemotactic (Ming, Bersani et al. 1987). TNFα is capable of inducing gene expression, apoptosis and necrosis, which illustrates the central role of this molecule in cell function and physiology (Wajant, Pfizenmaier et al. 2003). TNFα is central to the innate immune response to pathogens and injury (Kollias and Kontoyiannis 2002; Pfeffer 2003).

1.7.1.4 Transforming Growth Factor Beta-1 (TGFβ-1)

TGFβ-1 is a polypeptide member of the transforming growth factor beta superfamily of ligands. The secreted protein is a key regulator in immune defense, serving roles in both the innate and adaptive components (Wahl 1992; Wahl and Chen 2003; Wahl and Chen 2005; Li, Wan et al. 2006). TGFβ-1 is involved in a number of cellular

processes including proliferation, differentiation, cell death and the production of cytokines (Shi and Massague 2003). Given this repertoire of functions it makes sense that TGFβ-1 and other family members are highly expressed in the endometrium (Jones, Stoikos et al. 2006). TGFβ-1 is expressed in both the sromal and epithelial cells of the endometrium (Gold, Saxena et al. 1994; Godkin and Dore 1998), however, there doesn't appear to be any change in expression across the menstrual cycle. TGFβ-1 induces the upregulation of leukaemia inhibitory factor (LIF), a cytokine suggested to be important for implantation (Perrier d'Hauterive, Charlet-Renard et al. 2005).

TGFβ-1 is also an important factor in the modulation of endometrial immune response including mediating immune tolerance of seminal plasma and the allogeneic conceptus in mice (Schmidt-Weber and Blaser 2004; Jones, Stoikos et al. 2006). TGFβ-1 levels are upregulated in the endometrium when the concentration of progesterone is increased and downregulated during progesterone withdrawal (Casslen, Sandberg et al. 1998). Members of the matrix metalloproteinase (MMP) family (1.7.3) are suppressed by progesterone and this may be via TGFβ-1, as a paracrine transducer (Bruner, Rodgers et al. 1995; Osteen, Keller et al. 1999; Osteen, Igarashi et al. 2003).

1.7.2 Chemokines

Chemokines are a group of specialised cytokine-like molecules which exhibit chemoattractive properties, and are involved with the promotion of leukocyte recruitment (Baggiolini 2001). Chemokines play a complex role in the immune

response with the induction of cell migration (Rossi and Zlotnik 2000). Chemokines and their receptors are classified into four families based on the motif displayed by the first two cysteine residues, figure 1.7.2.1. There are at least 17 CXC chemokines that bind to one or more of the CXC receptors (CXCRs), of which there are six (CXCR1-6). This group of chemokines includes IL-8 and IFN-γ-inducible protein (IP)-10. The CC chemokines currently have 28 members and includes macrophage inflammatory protein 1a, RANTES and eotaxins 1-3, and they bind to one or more of the ten CC receptors (CCR1-10). There is one CX3C chemokine (fractalkine or neurotaxin), which binds to the CX3CR1, which is currently the only receptor. There are two XC chemokines, including Lymphotaxin; this family has only one receptor, XCR1. In 2000 a new nomenclature system was proposed, in which each of the chemokines is designated as a numbered ligand for its respective receptor family, for example IL-8 is a CXC ligand and is designated CXCL-8, an update has also been recently published (Prieschl, Kulmburg et al. 1995; Hortsch 1997; Murphy, Baggiolini et al. 2000; 2001; 2002; Bacon, Baggiolini et al. 2002; 2003). A table showing the chemokine families with the new nomenclature, common names and the relevant receptors is included below (table 1.7.2.1).

The cells of inflamed tissue are capable of the release of a variety of chemokines, and upon infection with bacteria or viruses, the chemokines are involved in the recruitment of immune effector cells. There is a tight interplay between cytokines and chemokines in the determination of the specific immune response required (Bisset and Schmid-Grendelmeier 2005). Chemokines can be increased or inactivated by tissue proteases, such as MMPs (McQuibban, Butler et al. 2001).

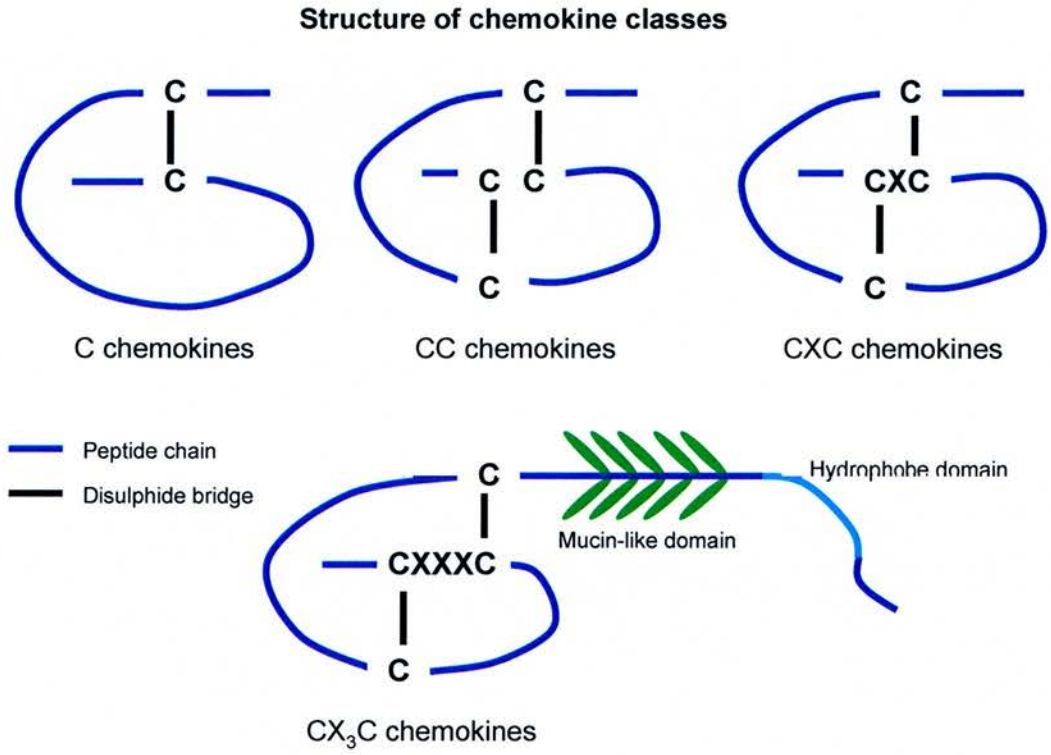


Figure 1.7.2.1 A schematic of the four chemokine classes based on the the positioning of conserved cysteine residues and disulphide bonds. The CX_3C chemokine also has a hydrophobe domain and a mucin-like domain.

CC chemokines			CXC chemokines		
Official name	**Other name(s)**	**Receptor**	**Official name**	**Other name(s)**	**Receptor**
CCL1	I-309, TCA-3	CCR8	CXCL1	Gro-α, GRO1, NAP-3	CXCR2
CCL2	MCP-1	CCR2	CXCL2	Gro-β, GRO2, MIP-2α	CXCR2
CCL3	MIP-1α	CCR1, CCR5	CXCL3	Gro-γ, GRO3, MIP-2β	CXCR2
CCL4	MIP-1β	CCR5	CXCL4	PF-4	
CCL5	RANTES	CCR1, CCR3, CCR5	CXCL5	ENA-78	CXCR2
CCL6	C10, MRP-2		CXCL6	GCP-2	CXCR2
CCL7	MARC, MCP-3	CCR1, CCR2, CCR3	CXCL7	NAP-2, CTAPIII, β-Ta, PEP	CXCR2
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5	CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR1, CXCR2
CCL9/CCL10	MRP-2, CCF18, MIP-1γ	CCR1	CXCL9	MIG, CRG-10	CXCR3
CCL11	Eotaxin	CCR3	CXCL10	IP-10, CRG-2	CXCR3
CCL12	MCP-5	CCR2	CXCL11	I-TAC, β-R1, IP-9	CXCR3
CCL13	MCP-4, NCC-1, Ckβ10	CCR1, CCR2, CCR3	CXCL12	SDF-1, PBSF	CXCR4
CCL14	HCC-1, MCIF, Ckβ1, NCC-2, CCL	CCR1	CXCL13	BCA-1, BLC	CXCR5
CCL15	Leukotactin-1, MIP-5, HCC-2, NCC-3	CCR1, CCR3	CXCL14	BRAK, bolekine	
CCL16	LEC, NCC-4, LMC, Ckβ12	CCR1	CXCL15	Lungkine, WECHE	
CCL17	TARC, dendrokine, ABCD-2	CCR4, CCR8	CXCL16	SRPSOX	CXCR6
CCL18	PARC, DC-CK1, AMAC-1, Ckβ7, MIP-4		CXCL17	DMC, VCC-1	
CCL19	ELC, Exodus-3, Ckβ11	CCR7	**CX_3C chemokines**		
CCL20	LARC, Exodus-1, Ckβ4	CCR6	**Official name**	**Other name(s)**	**Receptor**
CCL21	SLC, 6Ckine, Exodus-2, Ckβ9, TCA-4	CCR7	CX3C1	Fractalkine, Neurotactin, ABCD-3	CX_3CR1
CCL22	MDC, DC/β-CK	CCR4	**C chemokines**		
CCL23	MPIF-1, Ckβ8, MIP-3, MPIF-1	CCR1	**Official name**	**Other name(s)**	**Receptor**
CCL24	Eotaxin-2, MPIF-2, Ckβ6	CCR3	CL1	Lymphotactin α, SCM-1α, ATAC	XCR1
CCL25	TECK, Ckβ15	CCR9	CL2	Lymphotactin β, SCM-1β	XCR1
CCL26	Eotaxin-3, MIP-4α, IMAC, TSC-1	CCR3			
CCL27	CTACK, ILC, Eskine, PESKY, skinkine	CCR10			
CCL28	MEC	CCR10			

Table 1.7.2.1 Table adapted and updated based on data from Janeway et al. (Janeway, Travers et al. 2001).

1.7.2.1 Interleukin – 8 (IL-8; CXCL-8)

IL-8, also known as neutrophil-activating peptide-1 and is secreted by a number of different cell types in response to immune stimuli. These cell types include, NK cells (Saito, Kasahara et al. 1994; De Sanctis, Blanca et al. 1997; Marti, Bertran et al. 2002), neutrophils (Fujishima, Hoffman et al. 1993), monocytes (Abe, Kobayashi et al. 1991), fibroblasts , endothelial (Gimbrone, Obin et al. 1989) and epithelial cells.

Within the endometrium IL-8 has a role in angiogenesis and the induction of stromal cell proliferation (Arici, Seli et al. 1998) and is produced by both ESCs and endometrial epithelial cells (EECs) in culture. However, there appears to be little or no expression of IL-8 by the stromal cells *in vivo* (Critchley, Jones et al. 1999). IL-8 has been localised to both surface and glandular epithelium, as well as to the perivascular cells (Critchley, Kelly et al. 1994; Jones, Kelly et al. 1997). The expression of IL-8 is modulated via the inflammatory cytokines TNFα and IL-1β (Arici, Seli et al. 1998). It has also been demonstrated that IL-8 is upregulated in response to progesterone withdrawal *in vivo* (Critchley, Jones et al. 1999). IL-8 levels have been found to be maximal during the late secretory and menstrual phase of the menstrual cycle (Lam, Briton-Jones et al. 2004). The expression of IL-8 during the late secretory phase is believed to be involved with the recruitment of neutrophils which are known to invade the endometrium prior to menstruation (Lam, Briton-Jones et al. 2004).

1.7.3 Matrix Metalloproteinases (MMPs)

The actions of MMPs were first observed during frog metamorphosis where the enzymes were involved in the resorption of the tail, this was later identified as collagenase (Curry and Osteen 2003). The MMPs are a family proteases, that have been described as being zinc and calcium dependent endopeptidases that function to maintain the ECM (Egeblad and Werb 2002). MMPs are regarded as multifunctional enzymes due to the range of subtrates and have been identified to have a role in processes such as embryogenesis, angiogenesis, wound repair and tissue remodelling. MMPs also have the ability to interact and modulate cytokines and growth factors (McCawley and Matrisian 2001), suggesting a role in the immune response. MMPs and their inhibitors, TIMPs also have an important role in the female reproductive tract (Salamonsen 2003; Kaitu'u, Shen et al. 2005).

1.7.3.1 MMPs in menstruation

The matrix metalloproteinases (MMPs) are enzymes which are released by the infiltrating immune cells and by the stromal cells. MMPs have also been implicated with the breakdown of the extracellular matrix (Schatz, Krikun et al. 1997; Lockwood, Krikun et al. 1998; Salamonsen and Woolley 1999). The role of MMPs in menstruation is characterised by the timing of their expression within the endometrium (Rodgers, Osteen et al. 1993; Rodgers, Matrisian et al. 1994; Li, Post et al. 2000). Some at least of the MMPs are presumed to be under the control of progesterone. The withdrawal of progesterone from endometrial stromal cells

(ESCs) in culture showed an enhancement in the production of pro-MMP-2 (Irwin, Kirk et al. 1996) and pro-MMP-3 (stromelysin-1) (Schatz, Papp et al. 1994; Lockwood, Krikun et al. 1998). This was further supported with human ESCs in culture mimicking the luteal phase, MMP-1, 2, 3 and 4 in latent form were upregulated with progesterone withdrawal (Salamonsen, Butt et al. 1997). The MMP tissue inhibitors (TIMPs) were also investigated with respect to the withdrawal of progesterone, however, no regulation of TIMPs 1, 2 or 3 was observed. This is an indication that the regulation of MMPs is more complex than simply being progesterone regulated. Leukocytes also provide a source of MMPs and their control may be via the paracrine interaction between the stromal and epithelial cells (Salamonsen and Lathbury 2000).

1.7.3.2 Matrilysin (MMP-7)

Matrilysin is one of the smallest members of the MMP family, 28 kDa and was first discovered in the uterus of the rat and designated the name "pump" for putative uterine metalloproteinase (Woessner 1996). Recently, there has been much research interest in the role for matrilysin in mucosal immunity. In contrast to the expression of other MMPs, it has been demonstrated that matrilysin is expressed in non-damaged mucosa as well as part of the inflammatory process in the event of injury or infection. It has also been recognised that MMP-7 is constitutively expressed across mucosal areas and specifically those associated with vulnerability to infection as they are exposed to the environment such as the lining of the gut (Wielockx, Libert et al. 2004). However, this was shown in the rodent and it was suggested that this was not the case in humans as there is an absence of MMP-7 in the gut. Wilson *et al*

(Wilson, Ouellette et al. 1999) reported the co-expression of matrilysin and pro-α-defensins in the paneth cells of the murine gut, suggesting that the enzyme has a role in regulating the activity of defensins. In vitro studies revealed that matrilysin can cleave the pro-peptides into their mature and active forms. Mouse knockouts for matrilysin showed a deficiency in producing active antimicrobials and further leaving the animals vulnerable to exogenous microbes (Wilson, Ouellette et al. 1999). However, later work demonstrated the cleavage of the pre-form of human defensin 5, by a murine intestinal trypsin into its active form. Matrilysin was shown to cleave HD5 but, in the wrong place (Ghosh, Porter et al. 2002). The role for matrilysin in the cleavage of pro-peptides in the human has been dismissed as it has not been detected within the gut (Wielockx, Libert et al. 2004). However, matrilysin has been found to be expressed in the female reproductive tract (Rodgers, Osteen et al. 1993).

1.8 Menstruation as an inflammatory event

Menstruation occurs in the event of implantation failure, and results in the functional layer of the endometrium being shed. The overall process occurs in response to the declining progesterone levels that results from the demise of the corpus luteum. However, the process is described as being multi-factorial, where the hormonal influence is only a part of the story (Jabbour, Kelly et al. 2006). The premenstrual withdrawal of progesterone initiates the upregulation of inflammatory mediators such as cytokines, chemokines, matrix metalloproteinases (MMPs) and the influx of leukocytes. The presence and active role of these immune cells and factors have led

to the process of menstruation being described as an inflammatory event (Finn 1986; Kelly, Illingworth et al. 1994; Critchley, Kelly et al. 2001).

1.8.1 Leukocytes within the female reproductive tract

The leukocyte population is composed of macrophages, uterine Natural Killer (uNK) cells and neutrophils. These cells can be modulated indirectly via the sex steroid hormones, in the absence of classical nuclear receptors this is believed to be mediated via paracrine factors (Poropatich, Rojas et al. 1987; Henderson, Saunders et al. 2003). However, it is also possible for sex steroid responsiveness to be mediated via the glucocorticoid receptor (GR) binding of progesterone (Wira, Fahey et al. 2005). Neutrophils are present in low numbers within the endometrium across the cycle. Their numbers are however, greatly increased immediately prior to the onset of menstruation, similar to that described in the event of inflammation (Noyes, Hertig et al. 1950; Kamat and Isaacson 1987; Poropatich, Rojas et al. 1987). In the event of pregnancy there is a large number of large granular lymphocytes (LGL), and represent approximately 70% of the total number of leukocytes present in the 1st trimester with a subsequent decline during the 2nd trimester (Loke, King et al. 1995), thus, indicating a role for these cells in the process of implantation and placentation, however, their exact role has still to be defined (Loke, King et al. 1995; King, Burrows et al. 1998; King 2000). However, the presence of an infection or an over-zealous immune response would clearly be harmful to the pregnancy. The presence of high levels of NK cells within endometrium is associated with a greater incidence of pregnancy loss (Yamada, Morikawa et al. 2003; Carp 2004; Shimada, Kato et al.

2004). In the absence of pregnancy the number of NK cells decline, however, there are conflicts within the literature as to whether these cells undergo apoptosis (Jones, Bulmer et al. 1998; Jones, Searle et al. 1998; King 2000; Pongcharoen, Bulmer et al. 2004), this is discussed further in section 1.8.5 of the current thesis. A table (1.8.1.1) summarising the leukocyte populations present in the pregnant and non-pregnant endometrium is detailed below.

	Non-pregnant endometrium		**Early decidua**	
	Proliferative	Secretory	Basalis (Trophoblast$^+$)	Parietalis (Trophoblast$^-$)
Granulocytes				
Neutrophils	-[a]	-/+[b]	-/+	-
Eosinophils	-	-	-	-
Basophils	-	-	-	-
Lymphocytes				
B Cells	- (+)[c]	- (+)	- (+)	- (+)
T Cells	+[d]	+	+	+
NK Cells (LGL)	+	+++[e]	+++++	+++
Macrophages	+	+	+++	+

Table 1.1.8.1 Summary of the presence and timing of the leukocyte populations in the endometrium from (King, Burrows et al. 1998). Letters: a – indicates no cells; b – variable numbers of cells; c – variable, low density of cells; d – high number of cells present; e - ≥ +++ indicates the density of cells present.

1.8.2 Neutrophils and eosinophils

In the lower reproductive tract the role of the neutrophil is to prevent the cervical and vaginal regions from becoming infected, the phagocytosis of dead and damaged sperm; and cervical maturation during parturition (Smith, Wira et al. 2006). In the

upper reproductive tract the neutrophils are believed to have an additional role in the breakdown and remodelling of tissue. They are present in very low numbers in the endometrium until immediately prior to the onset of menstruation when the numbers can increase and comprise up to 15% of the total cell number (Salamonsen and Lathbury 2000).

It has recently been suggested that the neutrophils localised to the Fallopian tube exhibit a different phenotype from those within peripheral blood (Smith, Wira et al. 2006). Smith et al (Smith, Wira et al. 2006), suggest that this difference in phenotype enables a more rapid innate immune response, in protection of both the Fallopian tube and the peritoneal cavity.

Eosinophils exhibit a similar pattern of occurrence as described for the neutrophils in the cyclic endometrium. There is an increase in the number of these cells at menstruation, where they form aggregations and can make up around 5% of the total number of cells at this time (Poropatich, Rojas et al. 1987; Salamonsen and Lathbury 2000).

1.8.3 Lymphocytes

The lymphocyte population consists of B-lymphocytes and T-lymphocytes and are found within aggregates in the basal layer of endometrium. They are present throughout the menstrual cycle and in relatively low numbers (Bulmer, Longfellow et al. 1991). The number of B-lymphocytes does not vary, whilst T-lymphocytes

numbers are slightly elevated perimenstrually. T-lymphocytes have also been identified within the functional layer of endometrium and intraepithelial sites (Loke, King et al. 1995).

Approximately 85% of the T cells are positive for the αβ T cell receptor (TCR^+), with the remainder of the endometrial population identified as γδ TCR^+ (Chen, Huang et al. 1995). The γδT cells and αβT cells are members of different components of the immune system, the innate and adaptive respectively (Hayday, Theodoridis et al. 2001). The γδT cells are a component of the innate immune system and are responsible for the recognition of antigens, allogens and self-antigens as part of the first line of defence (Mincheva-Nilsson 2003). The presence of these cells may be to regulate the adaptation of the maternal immune system to the presence of a semiallogeneic embryo (Mincheva-Nilsson, Hammarstrom et al. 1992; Meeusen, Bischof et al. 2001). The function of TCR^+ cells in the endometrium may be as immunosuppressors with or without cytolytic activity, however, it remains unconfirmed (Suzuki, Hiromatsu et al. 1995; Mincheva-Nilsson, Nagaeva et al. 2000). There are greater numbers of $CD8^+$ cells relative to the number $CD4^+$ cells within the endometrium than the ratio observed in peripheral blood. The $CD8^+$ cells exert cytolytic activity during the proliferative phase of the menstrual cycle; this activity ceases at the onset of the secretory phase (White, Crassi et al. 1997; Yeaman, Guyre et al. 1997).

B cells have been characterised in the mucosa of the gut and lung, and function to produce IgA antibodies in providing immune protection. There have been

descriptions of IgA synthesising plasma cells in the vagina, ectocervix and endocervix. The presence of plasma cells within the endometrium has been considered to be an indication of endometritis (Buckley 1989). However, recently this view has been questioned with reports of plasma cells within healthy endometrium (Achilles, Amortegui et al. 2005).

1.8.4 Macrophages

The macrophages have been identified to be widely distributed in the female reproductive tract and are representative of approx. 10% of the total number of leukocytes in the female reproductive tract (Givan, White et al. 1997). The changes in steroid hormone levels appear to be coincident with macrophage migration, especially within the endometrium (Jones, Kelly et al. 1997; DeLoia, Stewart-Akers et al. 2002). The cyclic variation in macrophage numbers may be as an indirect consequence of steroid hormone mediated changes in the levels of circulating cytokines and chemokines (Wira, Fahey et al. 2005), (table 1.1.8.1). The majority of endometrial macrophages express major histocompatibility complex (MHC) class I or class II (Bulmer, Lunny et al. 1988), suggesting a role as antigen presenting cells (APCs). Macrophages are responsible both for the secretion of and response to cytokines, and have a pivotal role in the inflammatory response (Wira, Fahey et al. 2005).

1.8.5 Natural Killer cells

Natural Killer (NK) cells are large granular lymphocytes (LGL) and have a pivotal role as part of the innate immune system, exerting effects on both immune and non-immune cells. They have the ability to kill tumour cells and are responsible for the secretion of a number of cytokines. They have been described as being sentinels of the innate immune response having the ability to attack pathogens and they can upregulate other immune effectors.

NK cells express the cell surface antigens CD56, an isoform of NCAM (neural cell adhesion molecule) and most express the low affinity Fc-γ receptor, CD16. NK cells are found in peripheral blood, lymphoid organs, liver and mucosal tissues including the uterus. The cells within the blood have been divided into two classes and this has been based on the expression density of the surface antigen CD56. In the blood over 90% of the cells are described as being $CD56^{dim}$. This subset of cells has been shown to have a high level of lytic activity against tumour cells, and are further characterised by the expression of CD16 (FcRγIII), and killer cell Ig-like receptors (KIRs). Many of these cells also express CD57 (Jacobs, Hintzen et al. 2001; Wendt, Wilk et al. 2006). The remainder of NK cells are described as being $CD56^{bright}$ and have the ability to produce cytokines when stimulated by monokines (Jacobs, Hintzen et al. 2001; Cooper, Fehniger et al. 2004; Wendt, Wilk et al. 2006). Within the female reproductive tract the NK cells have been shown to have a distinct phenotype from that of peripheral blood, and have been described in the literature as endometrial granulocytes, granular endometrial stromal cells and decidual NK (dNK) cells (Bulmer, Longfellow et al. 1991). NK cells of the uterus are $CD56^{bright}$ and

most lack CD16. Uterine NK (uNK) cells also express KIRs, CD9 and CD69 on their cell surface (Verma, King et al. 1997; Koopman, Kopcow et al. 2003; Eriksson, Meadows et al. 2004; Eriksson, Meadows et al. 2006).

Uterine NK cells are present in low numbers throughout the menstrual cycle. However, there is a dramatic increase in their numbers during the secretory phase (Bulmer, Longfellow et al. 1991; Loke, King et al. 1995; King, Burrows et al. 1996; Jones, Bulmer et al. 1998; King 2000). They form a major cellular component of the endometrial stroma and are occasionally located within the functional layer within intraepithelial sites.

1.9 Implantation

The process of implantation and endometrial receptivity is mediated by hormones, cytokines and growth factors (Carson, Bagchi et al. 2000). As the blastocyst arrives into the uterine cavity it is free floating and initiates the implantation process by establishing communication with the endometrium. A number of factors need to be in place in order for the hostile endometrium to be receptive and some of the mediators of this process are discussed below (1.9.1) (Achache and Revel 2006). Once dialogue is established there follows a series of synchronised events. The next step is apposition, where the blastocyst becomes loosely attached to the wall of the endometrium. This allows for local paracrine signalling which gives rise to a stronger attachment. This stronger attachment allows the trophectoderm cells from the blastocyst to migrate through the epithelial cells of the endometrium, and into the basement membrane. This is followed by the trophoblastic invasion of the stroma.

If the endometrium is not receptive to taking part in any of these events then implantation will fail (Norwitz, Schust et al. 2001; Sharkey and Smith 2003). The receptive endometrium has been characterised at the histological level which includes the increased activity of the secretory glands, an increase in vascularity and the development of pinopodes (Norwitz, Schust et al. 2001). This brief progesterone dependent appearance of pinopodes has come to be regarded as an indicator of a receptive endometrium, typically days 20 – 24 of the menstrual cycle (Bergh and Navot 1992; Norwitz, Schust et al. 2001). The role of the steroid hormones in the mediation of implantation has been subject to much research, however, much of the molecular mechanisms remain to be determined (Norwitz, Schust et al. 2001).

1.9.1 Mediators of endometrial receptivity

Uterine preparation for implantation involves the synchronisation of a number of cytokines, steroid hormones, growth factors, adhesion molecules and other factors that have been identified as important for the preparation of the endometrium for implantation (Beier-Hellwig, Sterzik et al. 1994; Beier and Beier-Hellwig 1998; Achache and Revel 2006). As with the pinopodes mentioned above a number of markers for endometrial receptivity have been identified and many of these are subject to regulation by the steroid hormones (Lessey, Yeh et al. 1996; Lessey 2003). During the secretory phase, the endometrium undergoes a number of morphological changes under the influence of progesterone and differentiates into decidua. The decidualisation of the stroma and increase in secretory glandular epithelium is essential for the establishment of pregnancy (Loke, King et al. 1995; King 2000; Achache and Revel 2006). The glandular epithelium secretes a number of

hormonally regulated proteins such as albumin and glycodelin[1]. Progesterone also functions as an antagonist for oestrogen directed proliferation. The inhibition of oestradiol levels results from the downregulation of ERα by progesterone which acts as an antagonist (Lessey 2003). This downregulation of ERα also indirectly results in an increase in expression of integrins which is inhibited by oestradiol. Another example of indirect regulation by progesterone is via the direct increase in the expression of calcitonin (Ding, Zhu et al. 1994; Kumar, Zhu et al. 1998; Zhu, Cullinan-Bove et al. 1998), which increases the level of intracellular calcium and this allows for the regulation of E-cadherins. Progesterone increases the amount of osteopontin and its receptor, alphavbeta (3) ($\alpha_V\beta_3$) integrin expression (Apparao, Murray et al. 2001; Johnson, Burghardt et al. 2003; von Wolff, Bohlmann et al. 2004). The integrin $\alpha_V\beta_3$ and its ligand osteopontin has been suggested as a receptor for embryonic attachment (Lessey 2003). Progesterone may also regulate paracrine stromal factors such as epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF) which increase the epithelial expression of β3 integrins (Somkuti, Yuan et al. 1997; Lessey 2003), which are important for the formation of $\alpha_V\beta_3$. Another molecule associated with endometrial receptivity is MUC1, a member of the mucin family of glycoproteins. It is possible that MUC1 expression is downregulated in the presence of high levels of progesterone and that MUC1 is inhibitory to implantation (DeSouza, Surveyor et al. 1999). However, in the human it has been shown that the expression of MUC1 is increased from the proliferative to mid-secretory phase of the menstrual cycle (Hey, Graham et al. 1994; Aplin and Hey 1995). The data surrounding MUC1 expression in the human endometrium appears to be

[1] Glycodelin is also reported as progesterone associated endometrial protein, lactoglobulin, α-uterine protein, α-2 microglobulin, pregnancy associated α-2 globulin and placental protein 14.

contradictory. However, it has been suggested that there may be a local acting mechanism for the inhibition of MUC1 (Thathiah and Carson 2004). The surface of pinopodes are devoid of MUC1 molecules (Horne, Lalani et al. 2006), and this has been suggested to function by providing an area for embryo implantation. Meseguer *et al* have demonstrated that the site of blastocyst implantation is devoid of MUC1 molecules (Meseguer, Aplin et al. 2001), however, they also suggest that progesterone results in the upregulation of the molecule and that the blastocyst is responsible for the local downregulation. The role of the blastocyst in the mediation of MUC1 expression has also been demonstrated recently by Thathiah and Carson (Thathiah and Carson 2004).

1.10 The morphology and role of the Fallopian tubes

The Fallopian tube is a paired organ which bilaterally connects the peri-ovarian space with the uterus. This tubular organ has distinct regions extending and opening into the peritoneal cavity from the ovary to the uterus; namely the fimbria, ampulla and isthmus (figure 1.10.1 (A)). The convoluted nature of the lumen is demonstrated in a histological cross section (figure 1.10.1 (B)). From the centre these areas are characterised as the lumen, which is surrounded by the endosalpinx, myosalpinx and the mesosalpinx as the final layer. Each of these regions and layers demonstrate distinct morphologies which are representative of the distinct roles within the organ (Croxatto 2002). The endosalpinx is the mucosal layer and is made up of monostratified epithelia. The myosalpinx is made up of smooth muscle which is thick and thin at the isthmus and ampulla regions, respectively.

The ampulla exhibits a large surface area which is composed mainly of ciliated epithelia. In contrast the isthmus region has fewer folds and has epithelium which is predominantly made up of secretory cells. The fimbria functions in the catching of the oocyte after ovulation, prior to its transport along the ampullary region. This is the region in which the controlled arrival of the spermatozoa is mediated until the oocytes are fertilised. Following fertilisation, the zygote commences its pre-implantation development as it travels through the isthmus and on its way to the uterus. The morphology and function of the Fallopian tube is controlled via the ovarian steroids (Croxatto 2002). Ovarian endocrine mechanisms arise from the Graafian follicles as they prepare to ovulate, and functions to mediate the synchronisation of events required for successful fertilisation (Hunter and Rodriguez-Martinez 2004). There is a sequential gradient from the production of oestradiol from follicular secretions to that of progesterone resulting from the pre-ovulatory increase in the level of gonadotrophin (Hunter and Rodriguez-Martinez 2004). The increase in progesterone and decline in oestradiol has a major influence upon the structure and function of the Fallopian tubes. This ovarian endocrine influence is believed to be essential for the controlled capacitation of sperm, by ensuring timely presentation and favourable sperm:egg ratios (Hunter 1993; Hunter 1995; Hunter 1996). The influence of progesterone upon the epithelial cells of the Fallopian tube has also been documented (Hunter 1987; Mburu, Einarsson et al. 1996; Lefebvre, Lo et al. 1997; Mburu, Rodriguez-Martinez et al. 1997; Suarez and Pacey 2006). However, there is still little understood about some of the morphological changes that are assumed to take place within the human Fallopian tube.

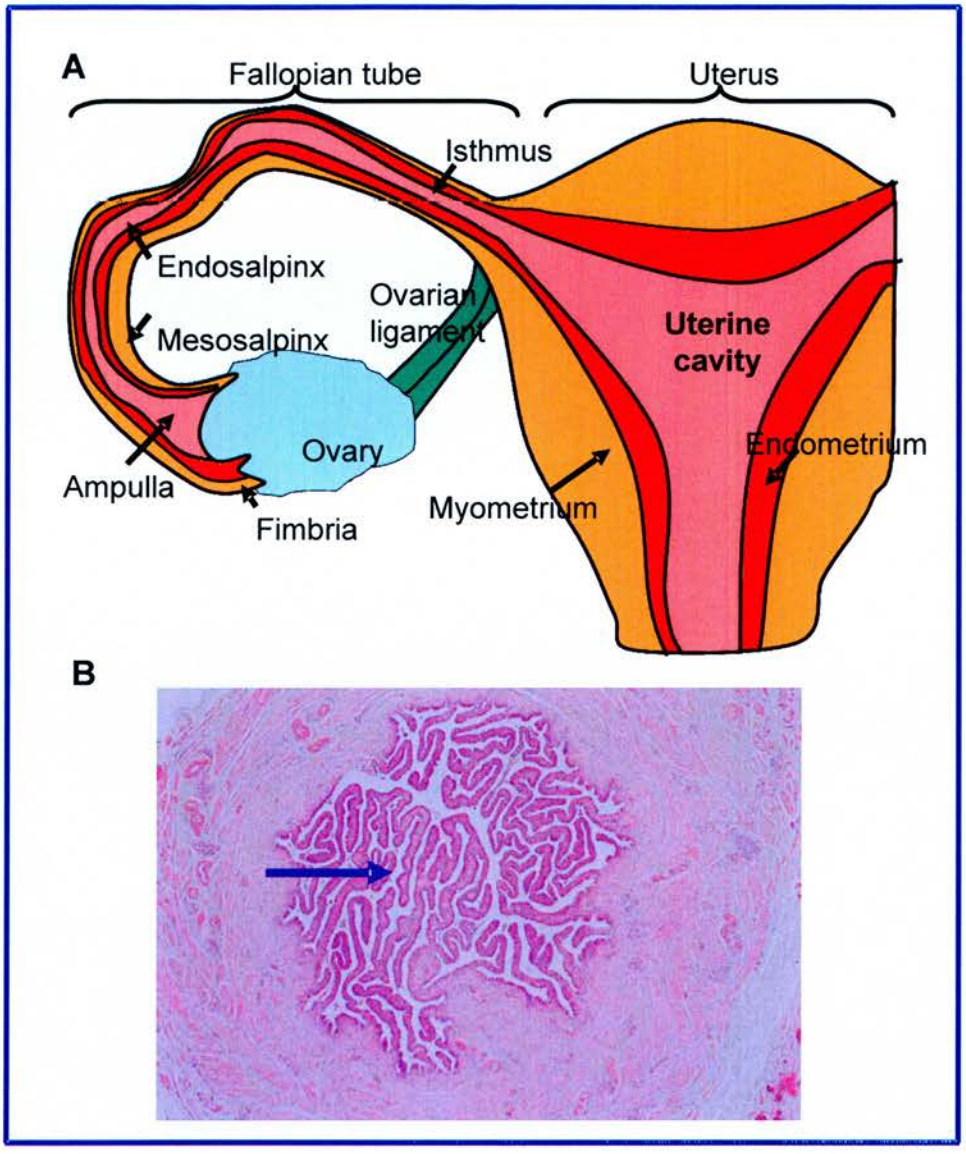


Figure 1.10.1 Schematic diagram illustrating the main structural components of the Fallopian tube in relation to the rest of the female reproductive tract (A). Haematoxylin and eosin staining of a cross-section of a Fallopian tube biopsy from the ampulla (B), the method used for the H&E staining is detailed in section 2.7.

1.10.1 The capacitation of spermatozoa and fertilisation

The capacitation of sperm within the female reproductive tract was first described as an important pre-fertilisation event 55 years ago (Schalkwijk, Chang et al. 1990). However, despite this early description and a significant amount of research into the area, much of the process is still poorly understood. A number of publications have served to both clarify and evaluate the sequential nature and physiological location of the steps involved in the capacitation of sperm. All of which is adequately reviewed by Hunter and Rodriguez-Martinez (Hunter and Rodriguez-Martinez 2004).

Much of the current knowledge of the sequential processes involved with the capacitation of sperm has resulted from observations made from *in vivo* timecourse experiments. These experiments have suggested that the uterus and the Fallopian tube function with synergism in the capacitation and preparation of sperm prior to fertilisation (Hunter 1969; Hunter and Hall 1974; Hunter and Hall 1974b; Lund, Alexopoulou et al. 2004). A number of publications have demonstrated that if sperm are placed directly into the Fallopian tube, capacitation takes longer than if the sperm is placed first within the uterus (Bedford 1970; Lund, Alexopoulou et al. 2004). The progression of sperm through the female reproductive tract allows for the actions of a variety of molecules and biochemistry to occur in a timely and sequential manner (Hunter and Rodriguez-Martinez 2004).

1.10.2 Transport of the ovum

Fertilisation commonly occurs in the ampullary region of the Fallopian tube where the nuclei of the egg and the sperm fuse to forming a diploid zygote. The zygote undergoes a series of cell divisions to form the morula. The zygote remains in the upper third of the female reproductive tract, normally for around 24 hours, before the cilliary and peristaltic action of the Fallopian tube moves the dividing zygote towards the uterine cavity (Croxatto 2002). The journey occurs over 4 – 5 days, during which the zygote will undergo a number of divisions under the constraint of the zona pellucida, causing the cells to get smaller with each division. By the time the resultant morula reaches the uterus, it will have divided into 8 – 16 daughter cells (blastomeres) (Croxatto 2002).

Upon arrival to the endometrial cavity, the blastomeres begin to polarise with the outer cells becoming flattened against the zona pellucida, whilst the inner cells continue to divide. Uterine fluid containing nutrients enters the morula and the blastocyst is formed (Pulkkinen 1995; Croxatto 2002).

Following fertilisation the zona pelucida serves to prevent the blastocyst from adhering to the Fallopian tube wall. However, upon arrival to the uterus and before implantation can occur the blastocyst must "hatch" from this protective layer (Mastroianni 1999). The degradation of the zona pelicuda is thought to be brought about via the secretions of both the blastocyst and the uterus. After the shedding of this protective, glycoprotein layer the blastocyst is ready to commence the process of implantation (Edwards 1994; Mastroianni 1999).

1.10.3 Ectopic pregnancy

Ectopic pregnancy is an important cause of maternal death during the first trimester of pregnancy (RCOG 2004). Sites of ectopic gestation include the Fallopian tube, cervix, ovary and abdominal cavity (figure 1.10.3.1). Over 95% of ectopic pregnancies occur in the Fallopian tube, 70% occur in the ampulla, 12% in the isthmus, 11% in the fimbria, and 2% in the interstitial/cornual region (figure 1.10.3.1) (Breen 1970; Bouyer, Coste et al. 2002; Corpa 2006). The precise mechanisms and causes of an ectopic implantation are not clearly understood, however, a number of risk factors have been identified (Farquhar 2005). Some of the main risk factors include, previous tubal surgery, genital infection and smoking (Farquhar 2005). The impairment of tubal transport is widely thought to be a major cause of tubal pregnancy (Corpa 2006), and some of the causes are discussed in more detail in chapter 6.

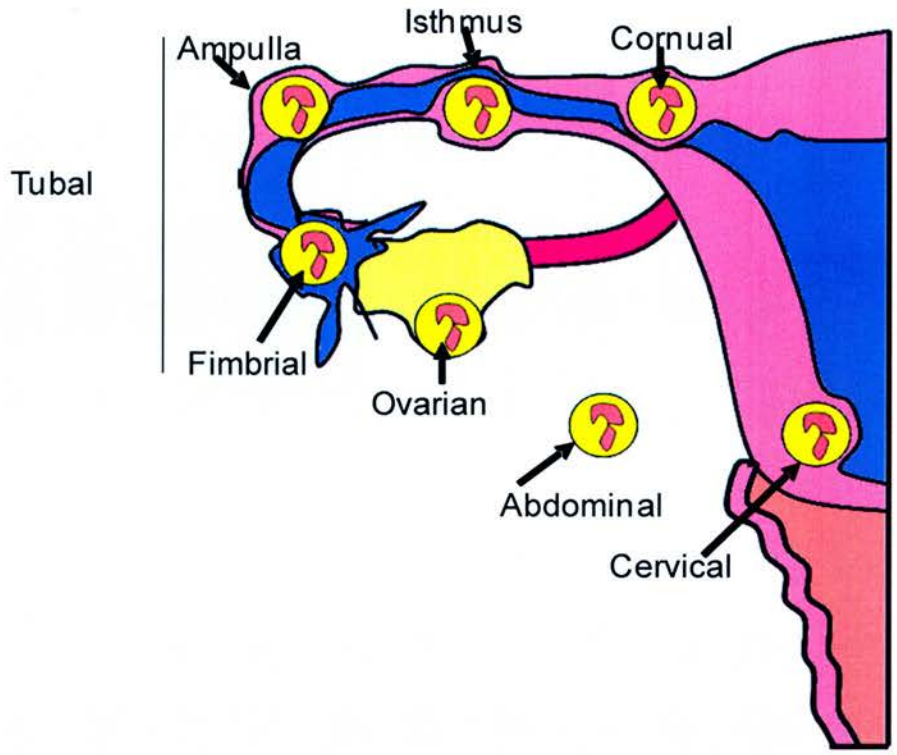


Figure 1.10.3.1 Diagrammatic demonstration of the documented sites of ectopic pregnancy. The figure was redrawn and adapted from Corpa. (Corpa 2006).

1.11 Aims of the thesis

The female reproductive tract is potentially exposed to a wide range of pathogens and the risk of infection is thought to be variable across the menstrual cycle. The maintenance of an infection free reproductive tract is essential for the successful conception and maintenance of pregnancy. In common with other mucosal regions the female reproductive tract has a range of innate immune effectors including the recently identified natural antimicrobials. These peptides and proteins have been shown to be differentially expressed across the menstrual cycle giving rise to considerations for the possible distinct roles of these molecules. Their relationship with the sex steroids and with other immune factors is similary subject to cyclical variance in expression.

Natural antimicrobials are described as being rapid and short term acting members of the innate immune system and function as a bridge to the adaptive immune system. The timing of their expression may be relevant as a means of ascertaining their specific roles and possible interactions. The expression of these molecules in relation to time has not been investigated.

The effect of progesterone upon the expression of these molecules is apparent both in culture and in primary material. Progesterone has been shown to inhibit or increase the expression of others, such as human β-defensin 2 and secretory leukocyte protease inhibitor (SLPI), respectively. The precise nature of this effect has not been fully elucidated. The role of paracrine interaction between stromal and epithelial

components of the endometrium upon the expression of natural antimicrobials has also not been investigated.

The natural antimicrobials have been characterised in the cycling endometrium, cervix and vagina. However, there is comparatively very little described for the presence or actions of natural antimicrobials in the Fallopian tube. The Fallopian tube is the target for a number of sexually transmitted infections and thus has a requirement for an innate immune response. The influence of the ovarian cycle upon the expression of these molecules in the Fallopian tube has also to be elucidated.

In order to determine further the role of natural antimicrobials in the Fallopian tube, consideration of the effect of an ectopic implantation upon their expression would be useful. The causes of ectopic implantation are poorly understood and the role of innate immune effectors such as the antimicrobials may provide an insight into both the causes and implications. The effect of an ectopic implantation upon the uterus has not been fully elucidated and may offer opportunities for early diagnosis and the identification of candidate molecular markers. The expression of natural antimicrobials in the uterine decidua sampled from women with an ectopic gestation has not been described.

Thus, the aims of the thesis are:

1. To characterise the expression and regulation of a range of natural antimicrobials within the human endometrium using representative cell lines.
2. To investigate the role(s) played by the interaction between epithelial and stromal cells in the expression of these molecules.
3. To identify the interactions between the antimicrobials and with other innate immune effector molecules.
4. To elucidate the role of sex steroids in the regulation of natural antimicrobials.
5. To examine the expression patterns of these molecules over time in response to infection.
6. To describe the expression of antimicrobials in human Fallopian tubes with and without an ectopic gestation.
7. To examine the expression of natural antimicrobials in the uterine decidua of ectopic and failed intra-uterine pregnancy.

Chapter 2:

General Methods

Chapter 2:

Materials and Methods

Reagents and Materials are detailed in Appendix 1.

2.1 Sample Collection

Tissue samples to be used for immunohistochemistry were collected in Roswell Park Memorial Institute (RPMI) 1640 culture media. Some of the sample was fixed in 10% neutral buffered formalin (NBF) overnight at 4°C and subsequently stored in 70% ethanol until finally being embedded in wax. Tissue was also collected for PCR analysis, collected in RNA later and stored at -70°C until further processing as detailed in section 2.3. Finally, portions of tissue were also utilised for cell culture as detailed in section 2.2.

Written informed consent was obtained from all patients prior to biopsy collection and ethical approval was received from Lothian Research Ethics Committee (LREC/05/51104/12 and LREC 04/S1103/20). The details of which are provided below next to the applicable samples and further detailed within the relative data chapters within the current thesis.

2.1.1 Human uterine tissue

Endometrial biopsies were collected from women (18-45), undergoing gynaecological procedures for benign conditions. Samples were collected as full thickness (lumen to endometrial/myometrial junction) endometrial biopsies or with the use of an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France). All women reported regular menstrual cycles (25-35 days) and had not received any form of hormonal treatment in the three months prior to biopsy collection. Biopsies were dated according to stated last menstrual period (LMP) and were confirmed by histological assessment according to criteria of Noyes et al (Noyes, Hertig et al. 1950). Furthermore, circulating oestradiol and progesterone levels were measured at the time of biopsy collection and were consistent for both LMP and histological assignment of menstrual cycle stage. The concentration of oestradiol (E_2) and progesterone (P_4) were measured by radioimmunoassay (MRC HRSU assay lab).

First trimester decidua (8-11 weeks gestation) was obtained from patients (aged 18-45 years), undergoing surgical termination of pregnancy or surgical management of miscarriage and not received RU486. Decidual tissue collected during surgical management of an ectopic gestation was obtained with an endometrial suction curette (Pipelle).

2.1.2 Human Fallopian tube

Fallopian tube biopsies (n=10) were collected from fertile woman undergoing hysterectomy procedures for benign indications (age 18-45 years). The details of

these samples are provided in chapter 6 table 6.2.1.1. Fallopian tube biopsies (n=9) were in addition collected from women undergoing surgical management of ectopic pregnancy (age 25-40 years). The details of these samples are provided in chapter 6 in table 6.2.1.2.

2.2 Cell Culture

2.2.1 Separation of endometrial biopsies into glandular and stromal components.

The method utilised for the separation of the glandular and stromal components of the endometrium was adapted from that described by Osteen *et al.* (Osteen, Hill et al. 1989). The modifications that were made are outlined. The endometrial biopsies are washed 3 times in PBS and divided into smaller fragments. The fragments are immersed in collagenase (1 mg/ml), DNAase (0.1 mg/ml) and incubated for 80 minutes at 37°C. Culture media (RPMI 1640) was then added and the tissue disaggregated. This resulted with a suspension made up of single cells and larger glandular fragments. The suspension was centrifuged (3000 rpm, 3 minutes); the pellet resuspended with culture media and allowed to separate by the process of density sedimentation. The supernatant containing the stromal component was removed. Culture media was added and the density sedimentation step was repeated. The stromal cells were then placed into culture flasks with F' supplemented RPMI and maintained as in 2.2.2. The remaining glandular fraction was centrifuged as described previously. The pellet was resuspended in collagenase/DNAse, incubated

for 2 hour at 37°C. The suspension was centrifuged (3000 rpm, 3 minutes) and the resultant pellet resuspended in 50% matrigel.

2.2.2 Primary Endometrial Stromal Cells

Cells were cultured in complete RPMI (cRPMI) supplemented with basic fibroblast growth factor (bFGF) and oestradiol (10^{-8} M).

2.2.3 Cell lines

The human endometrial carcinoma derived cell line Hec-1A (human endometrial cancer-one) (Kuramoto, Tamura et al. 1972), was maintained in 75 cm^2 culture flasks (Corning Incorporated). The cells were grown in RPMI (Sigma) culture media supplemented with 10% fetal calf serum (FCS) (Mycoplex; PAA, Teddington, UK), L-glutamine (2 mM; Gibco BRL), penicillin (50 µg/ml; Sigma), streptomycin (50 µg/ml; Sigma) and gentamycin (5 µg/ml; Sigma). For treatment, cells were plated at a density of $5.2x10^5$ and grown overnight in 4 ml RPMI (2% FCS) in 6-well plates (Corning Incorporated). Treatments were added and incubated for an appropriate time (as described in data chapters). The culture supernatants were then removed and stored at -20 °C for analysis with ELISA. The cells were treated directly with Total RNA Isolation Reagent (TRIR; Abgene) for RNA extraction.

2.2.4 Treatment of Cells

Epithelial cells were seeded at $5x10^5$ cells per/well (6 well plate) and allowed to adhere overnight in 2% cRPMI (complete media). The cells were treated with a variety of mimics of infection (detailed in chapters 3, 4 and 8), in serum free conditions. Controls were grown under the same relevant culture conditions and were included throughout.

2.2.5 Co-Culture

In co-culture experiments endometrial stromal cells (ESCs) were seeded into 6 well culture plates and allowed to reach confluence. After attachment the media containing serum and hormones was removed and replaced with serum free RPMI. Hec-1A epithelial cells were seeded ($1.3x10^5$ cells/ml) into the interior of transwell culture inserts in 2% cRPMI and allowed to adhere. The inserts containing the epithelial cells were placed in the wells containing the stromal cells and the media replaced prior to treatment with inflammatory mimics with or without steroid hormones (detailed in chapter 4). Co-culture controls without treatment and single culture controls for both ESC and Hec-1A (treated and untreated) cells were included throughout. The co-culture set-up and methods of treatment are demonstrated in figure 2.2.5.1.

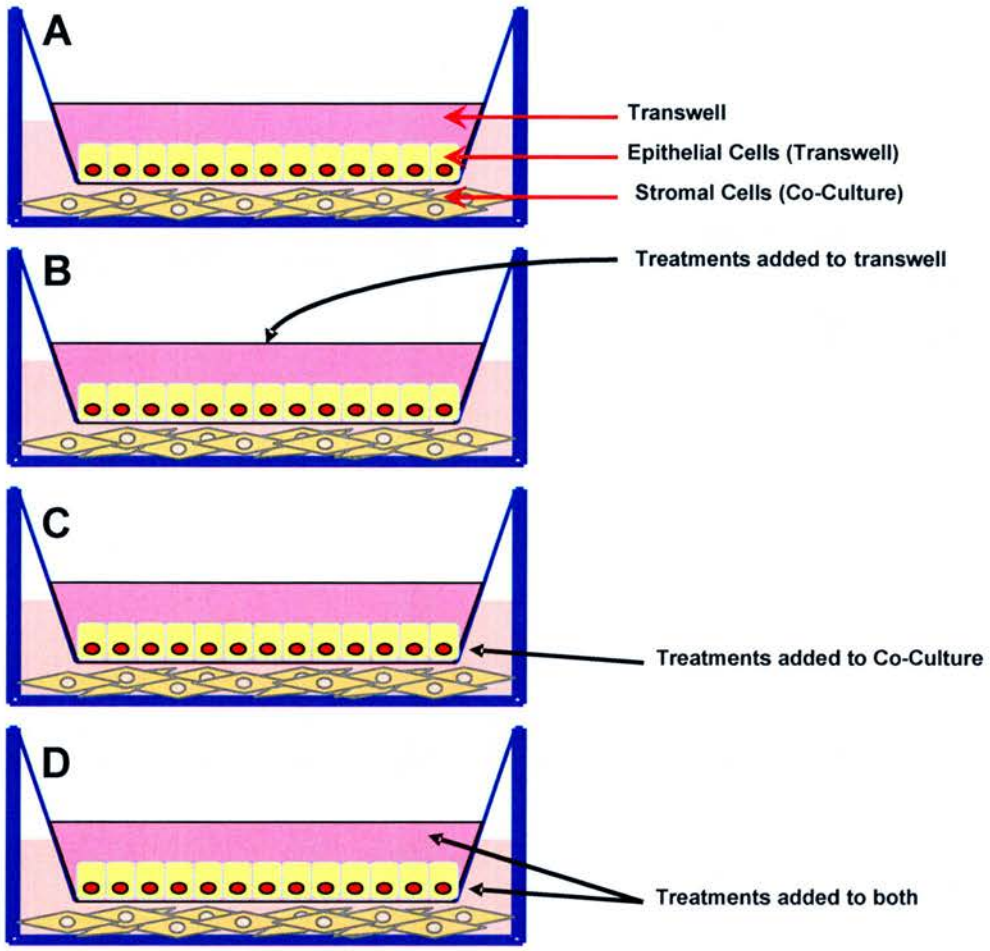


Figure 2.2.5.1 Co-Culture experiment set-up. This figure outlines the different set-up of culture plates for the investigation into the role of paracrine signalling in the expression of natural antimicrobials. (A) demonstrates the co-culture plate set-up; the epithelial cells were placed within the well insert – transwell. Whilst the stromal cells were plated within the original wells of the plate – these were described as co-culture cells. (B) The relevant treatments were added to the media contained within the transwell i.e. directly to the epithelial cells. (C) Depicts the media surrounding the stromal cells being treated. (D) Demonstrates the treatment of both components of the culture well set-up. All wells and transwells contained the same media with the exception of the relevant treatments where indicated.

2.2.6 Mycoplasma Screening and Treatment of Cells

There was regular screening and treatment of cultured cells for contamination with *Mycoplasma* spp. The maintenance of infection free culture conditions was essential in order to prevent unreliable data being obtained. A PCR-based method was employed to detect the commonest forms of mycoplasma. In the event that contamination was detected the cells were treated with plasmocin.

A mycoplasma test kit was obtained from Cambio Ltd. (Venor®GeM). Cell culture supernatant (100 µl) was heated at 95°C for 5 minutes and briefly centrifuged. The sample was then prepared for PCR as follows: 2 µl sample (dH_2O negative control; DNA template for mycoplasma positive control), 15.3 µl dH_2O, 2.5 µl 10x reaction buffer, 3 µl 50 mM $MgCl_2$, 2.5 µl primer/nucleotide mix, 2.5 µl internal control and 0.2 µl Taq polymerase. The reaction was carried out with the following conditions: 94°C for 2 minutes 1 cycle, 94°C 30 seconds, 55°C 30 seconds and 72°C for 39 cycles. The resultant products were analysed on an agarose gel (1.5 %, appendix II). A schematic summary of the protocol and an example of set of results is shown in figure 2.2.6.1.

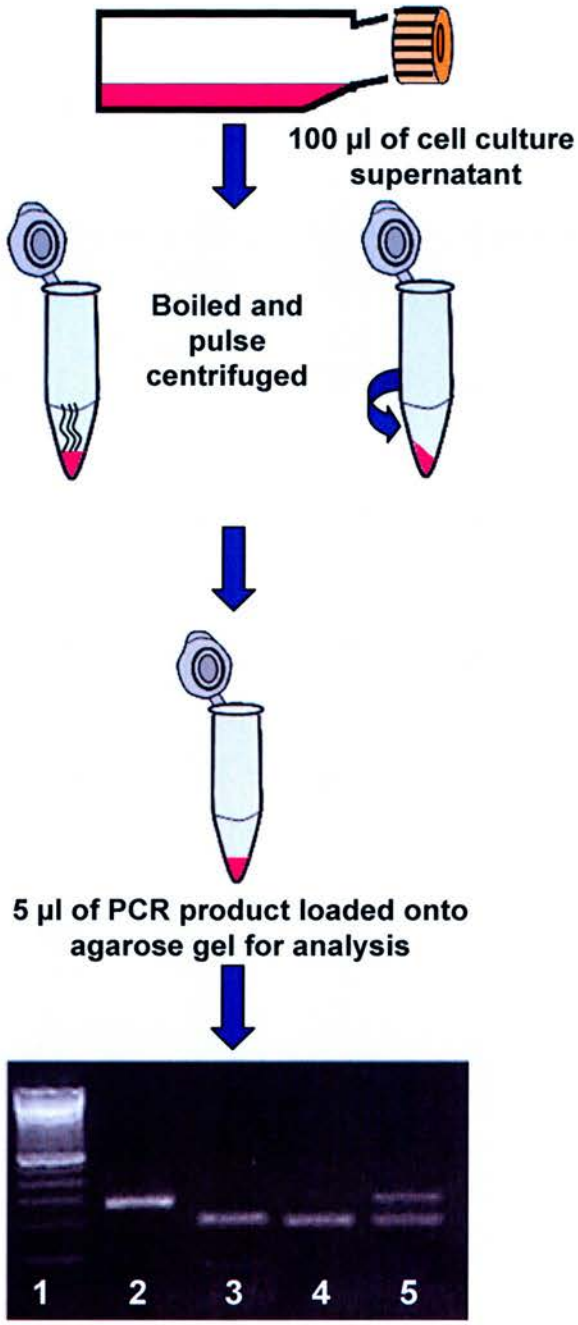


Figure 2.2.6.1 Schematic summary of the protocol utilised in the screening of cell culture stocks for the presence of *Mycoplasma* spp. Lane 1: 100 bp ladder; lane 2: positive control; lane 3: negative control; lane 4: non-contaminated sample and lane 5: contaminated sample.

2.3 RNA extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT-PCR).

2.3.1 RNA Extraction

(i) Total RNA Isolation Reagent (TRIR) and Phase Lock Gel Tubes (cell lines)

We extracted total RNA from the cell lines, which had been cultured in 6-well plates. 500 µl of TRIR was added to each of the wells and incubated on ice for 5 minutes to allow for cell lysis. The contents of the wells were then transferred into Phase Lock Gel (PLG) tubes (Eppendorf). Chloroform was added to each of the tubes at 0.2 x volume (100 µl) and the tubes shaken vigorously for 10 seconds and replaced on ice for a further 10 minutes. The tubes were placed in a centrifuge pre-cooled to 4 °C at 14000 rpm for 20 minutes. The organic phase containing protein and DNA becomes trapped beneath the gel layer, whilst the top aqueous phase containing the mRNA is poured off into clean 2 ml Eppendorfs. An equal volume of isopropanol was then added and the tubes inverted 2-3 times and replaced on ice and incubated for an hour. The tubes were centrifuged at 4 °C and 14000 rpm for 15 minutes. The isopropanol was removed and the resultant pellets washed with 70 % ethanol and centrifuged for a further 5 minutes and the remaining liquid removed. The pellets were dissolved in 25 – 50 µl RNA storage buffer (RSB; Ambion), determined by the size of pellet.

(ii) Qiagen RNA Extraction Columns (Tissue)

The RNeasy Mini Kit (Qiagen) was used and the extraction was performed according to the supplied protocol. The tissue was placed into a 2 ml tube containing 300µl of Buffer RLT with β-mercaptoethanol and a sterile stainless steel bead. The Qiagen tissue lyser was then used to homogenize the tissue at 30 Hz for 3 minutes. Nuclease free water (590 µl) and 10 µl of proteinase K solution was added to the homogenate and mixed with pipetting. The tubes were incubated at 55 °C for 10 minutes, before being centrifuged for 3 minutes at 10,000 rpm at room temperature. The resultant supernatant was poured off into an RNeasy mini spin column and the pellet discarded. Ethanol was added to the column and was centrifuged for 15 seconds at 10,000 rpm and the flow through was discarded. The column was washed with the addition of 350µl of buffer RW1 and centrifuged for 15 seconds at 10,000 rpm. This was followed by the addition of DNase treatment (10µl DNase I + 70µl Buffer RDD) and incubated at room temperature for 15 minutes, and then the column was washed as previously described. Two further washes with 500µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm; a final wash was added and centrifuged for 2 minutes. The RNeasy spin column was then transferred into a 1.5 ml collection tube and centrifuged at 14000 rpm for a further minute. For elution, 32 ul of nuclease free water was placed directly onto the RNeasy silica gel membrane after 5 minutes the tube was centrifuged for a further min at 10000 rpm. This step was repeated with the resultant eluant (RNA).

2.3.2 Reverse Transcription

The concentrations of the extracted RNA were determined using the formula: 260 nm value x dilution of RNA x 40 = RNA concentration (ng/µl). The concentration of the samples was adjusted with the addition of DEPC dH_2O (100 ng/µl).

The RNA samples were reversed transcribed using random primers with $MgCl_2$ (5.5 mmol/l), dNTPs (1 mmol/l), random hexamers (2.5 µmol/l), RNAse inhibitor (0.4 IU/µl) and Multiscribe reverse transcriptase (1.25 IU/µl). The resultant master mix was aliquoted 16 µl per tube with 400 ng template RNA (4 µl, 100 ng/µl). Mineral oil was added to the tubes and the PCR run with the following conditions: 20 minutes 25°C; 60 minutes 42°C and 95°C for 5 minutes. The resultant cDNA was diluted 2.5x with 1xTE buffer.

2.3.3 Real time quantitative polymerase chain reaction (Taqman; Q-RT-PCR)

This method of PCR utilises the release of a fluorescent reporter dye as a means of measuring the amount of PCR product obtained. This process is adequately reviewed and compared to that of conventional RT-PCR methods by Bustin, (Bustin 2000). Primers are used in order to detect the sequence of interest, and are concurrently used with a probe which targets the sequence specific to that between the annealed primers. The probe is labelled with two fluorescent dyes: a 5' reporter dye, FAM (6-carboxyfluorescein) and a 3' quencher dye, TAMRA (6-carboxytetramethylhodamine). The reporter dye on the probe utilised for the measurement of 18S is VIC in place of FAM. When the probe is annealed to the

target sequence the reporter dye is suppressed due to the quencher dye being in close vicinity. As the target sequence is amplified the probe is cleaved by the actions of the taq polymerase, separating the two dyes and thus, increasing the level of fluorescence as the quencher becomes distanced from the reporter dye. This increase in the level of fluorescence can be measured and is only detected if the target sequence has been amplified. The amount of specific amplicon is related to the amount of ribosomal 18S RNA and is constant to the amount of cDNA and acts as an internal control. It is possible to detect 18S concurrently within the reaction due to the different wavelength of fluorescence emitted by the VIC reporter dye.

A PCR mix (Applied Biosystems) containing Amperase is prepared with the addition of forward and reverse primers and probe (300 nmol/l) for the relevant sequence of interest. The primer and probes for the detection of 18S are also added (50 nmol/l). The resultant mixture is aliquoted into the requisite number of tubes and the relevant cDNA sample is added to each (2.5 µl/reaction replicate). 20 µl of this mixture is added in duplicate or triplicate to the wells of a 96 well PCR plate. For each plate the following controls are included: no template (dH_2O), a negative control from the experiment and a positive control where available. The plates are sealed with optical covers.

The reactions were carried out on ABI Prism 7900 (PE Biosystems) using a Taqman specific protocol. The conditions were as follows: 2 minutes 50°C; 10 minutes 95°C; followed with 40 cycles of the denaturing stage comprising 95°C 15 seconds and the

annealing/extension phase, 60°C 1 minute; the latter is also that of the data acquisition stage (figure 2.3.3.1).

The primers and probes were designed using PRIMER express software (PE Biosystems) and the sequences for all the primers and probes used are detailed in table 2.3.3.1

Amplicon	Forward Primer	Reverse Primer	Probe	Accession No.
Elafin	TGGCTCCTGCCCCATTATC	CAGTATCTTTCAAGCAGCGGTTA	ATCCGGTGCGCCATGTTGAATCC	AAB34627
Granulysin	CAGGGTGTGAAAGGCATCTCA	GGAGCATGGCTGCAAGGA	CGGCTGCCCCACCATGGC	NM006433.2
hBD1	TCAGCAGTGGAGGGCAATG	CCTCTGTAACAGGTGCCTTGAAT	CTCTATTCTGCCTGCCCGATCTTTACCA	NM005218
hBD2	CTGATGCCTCTTCCAGGTGTTT	CTGGATGACATATGGCTCCACTCT	AAGGCAGGTAACAGGATCGCCTATACCACCA	NM004942
hBD3	CAGAGGCGGCCGGTGT	CGAGCACTTGCCGATCTGTT	CTGTGCTCAGCTGCCTTCCAAAGGA	AF295370
hBD4	GGCAGTCCCATAACCACATATT	TGCTGCTATTAGCCGTTTCTCTT	TGTCCAATTCAAATTCGCTTCTCACTGGA	NP004933.1
hBD5	ACCTCAGGTTCTCAGGCAAGAG	AGAGGGACTCACGGGTAGCA	CTGCTATTGCCGAACCGGCCGT	AC134684.5
IL-1β	CGGCATCCAGCTACGAATCT	CATGGCCACAACAACTGACG	CGACCACCACTACAGCAAGGGCTTCAG	AC079753
IL-6	GCCGCCCCACACAGACA	CCGTCGAGGATGTACCGAAT	CCACTCACCTCTTTCAGAACGAATTGACAAAC	NM000600
IL-8	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGCAAAACTG	CCTTCCTGATTTCTGCAGCTCTGTGTGAA	M26383
MMP-7	GGATGGTAGCAGTCTAGGGATTAACTT	AGGAATGTCCCATACCCAAAGAA	CTGTATGCTGCAACTCATGAACTTGGC	Z11887
SLPI	GCATCAAATGCCTGGATCCT	GCATCAAACATTGGCCATAAGTC	TGACACCCCAAACCCAACAAGG	AF114471
TGFβ-1	CCCTGCCCCTACATTTGGA	AGGCGCCCGGGTTAT	ACACGCAGTACAGCAAGGTCCTGGC	X02812
TNFα	GGAGAAGGGTGACGACTCA	GGAGAAGGGTGACGACTCA	CGCTGAGATCAATCGGCCCGACTA	X02910

Table 2.3.3.1 Sequences and accession numbers of the primers and probes used within this thesis.

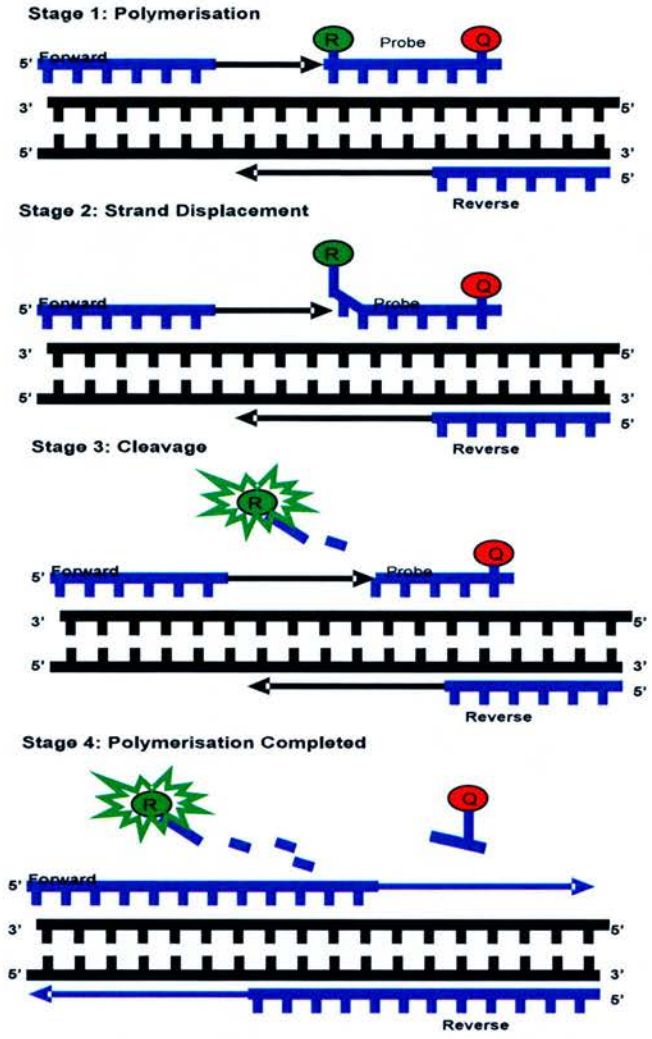


Figure 2.3.3.1 Quantitative Polymerase Chain Reaction (Taqman). ***Stage 1*** demonstrates the initiation of polymerisation. During this stage the probe anneals to the desired sequence and the reporter (R), FAM and quencher (Q), TAMRA dye are in close proximity to one another. The reporter dye is suppressed, resulting in a decrease in the amount of fluorescence detected. ***Stage 2*** the forward primer extends along the template which results in the displacement of the reporter dye of the probe. ***Stage 3*** Taq polymerase cleaves the probe breaking the link between the reporter and quencher dyes. This results in an increased amount of 'free' reporter dye (FAM), thus there is an increase in the level of fluorescence detected. ***Stage 4*** in the last stage the polymerisation reaction is completed. The amount of fluorescence detected will be proportional to the amount of PCR product generated. This will be representative of the initial quantities of the desired sequence. This figure and description has been adapted from the Taqman user's manual.

2.4 Enzyme Linked Immunosorbent Assays (ELISA)

2.4.1 Elafin ELISA

Elafin protein concentrations in cell culture supernatants were determined by ELISA. The method was adapted from the method described by Sallenave et al. (Sallenave *et al.* 1994). ELISA plates were coated overnight with anti-elafin IgG (1:500 in carbonate buffer, pH 9.6) at 4°C. The elafin antibody was an RIgG fraction (Sallenave *et al.* 1994). The plates were blocked, and samples/standards diluted in PBS (with 1% BSA and 0.05% Tween 20) were added to the plate and incubated for 2 hours at 37°C. The standards were added in duplicate and the range was from 0.4 – 100 ng/ml. The plates were then incubated with biotinylated anti-elafin IgG (2 µg/ml) for 2 hours, followed by streptavidin horseradish peroxidase (sigma) for a further 2 hours; both at 37°C. Plates were washed and chromogenic substrate 100 µl/well (2,2'-azino-bis-3-ethyl benz-thiazoline-6-sulfonic acid) with 0.001% H_2O_2 was added. Absorbance was measured at 490 nm on a microplate reader.

2.4.2 SLPI ELISA

The SLPI ELISA is a two site sandwich assay and has been described previously (King, Fleming et al. 2002). Throughout the assay all relevant dilutions were completed in SLPI ELISA buffer. ELISA 96 well assay plates were coated overnight at 4°C with goat-anti-SLPI antibody (2 µg/ml), 200 µl/well. The plates were blocked for 30 minutes at 37°C. Plates were washed 3 times with wash buffer prior to the

addition of the relevant standards and samples. The standards were added in duplicate and the concentration range was from 0.024 to 25 ng/ml of recombinant SLPI. On each plate 2 wells were included as controls for non-specific binding, containing 200 µl buffer. The plates were incubated at room temperature for 2 hours on a plate shaker. The plates were washed and incubated for a further hour with biotinylated mouse anti-human SLPI (1:10000), 200 µl/well. The plates were washed before the addition of streptavidin peroxidase (1:4000), 200 µl/well and incubated for 20 minutes at room temperature. The plates were again washed prior to the addition of the ELISA substrate, 200 µl/well. After 10 minutes or strong colour change, the reaction was stopped with the addition 50 µl/well of 2N sulphuric acid. The plates were read in a plate reader at 450 nm.

2.4.3 Chloramphenicol AcetylTransferase (CAT) Reporter ELISA

The CAT ELISA was used to measure the level of CAT expression in Hec-1A cells following transfection with the pCAT-reporter plasmid described in section 2.5. The sandwich ELISA quantitatively measures the amount of CAT enzyme expression as mediated via the elafin promoter in response to treatment as detailed in chapter 8.

Following treatment the cells were washed with chilled PBS and 1 ml of lysis buffer added to each well and incubated at 25°C for 25 minutes. The resultant supernatant was transferred into microfuge tubes and centrifuged for 15 minutes at 14,000 rpm and 4°C.

A calibration curve was prepared with a serial dilution of the stock enzyme. The standards and samples were placed into pre-coated micro-titre wells (200 µl/well) in duplicate. The wells were covered and incubated in the dark for 1 hour at 37°C. The samples/standards were removed and the wells washed 5 times with wash buffer, 250 µl/well, before the addition of anti-CAT-DIG 200 µl/well and incubated as before. The solution was removed and the wells washed as previously described, then 200 µl/well of anti-CAT-DIG-POD was added. The wells were incubated and washed as described before the addition of the POD substrate 200 µl/well. The wells were incubated in the dark at room temperature until sufficient colour development was observed (approximately 20 minutes). The absorbance was measured at 405 nm with a reference range of 490 nm, on a microplate reader.

2.5 Transfection of Hec-1A cells

2.5.1 Preparation of Plasmid DNA

The provision of *E. coli* glycerol stocks containing the plasmid constructs used in this thesis were a gift from Professor Jean-Michelle Sallenave of the Pasteur Institut, Paris.

The bacteria were grown in Luria Bertani (LB) medium containing ampicillin (appendix II), to a density of $4x10^9$ cells/ml after approx. 16 hours growth to ensure the transition from logarithmic phase to stationary phase growth. The extraction of plasmid DNA was carried out with a Qiagen Maxi-prep kit using the protocol

supplied with the following options. The cultured bacteria were harvested with centrifugation at 6000 xg for 15 minutes at 4°C and the resultant pellet was resuspended in 4 ml lysis buffer and vortexed. Then 4 ml buffer P2 was added to the suspension and mixed with inversion and incubated for 5 minutes at room temperature. The solution was precipitated with the addition of 4 ml of chilled buffer P3, mixed with inversion and incubated on ice for 15 minutes with periodic mixing. After incubation the solution was poured into QIAfilter cartridges and the flow through collected into a clean falcon tube on ice. QIAGEN-tips were allowed to equilibriate with the addition of buffer QBT prior to the addition of the supernatant, followed by the addition of wash buffer QC, 2 x 10 ml per tip. The DNA was eluted from the filter with the addition of 5 ml buffer QF, the eluate was collected into falcon tubes and precipitated with the addition of 3.5 ml of isopropanol at room temperature. The mixed solution was centrifuged at 15, 000 xg for 30 minutes at room temperature. The resultant pellet was washed twice with 70% ethanol, air dried and resuspended in TE buffer. The final concentration was measured using a spectrophotometer at 260 nm and checked on an agarose gel.

2.5.2 β-Gal reporter optimisation of transfection

Hec-1A cells were transfected with β-galactosidase reporter constructs in order to optimise the transfection efficiency.

Following transfection the cells were histochemically stained for β-galactosidase activity. The cells were stained using X-gal (5-Bromo-4-chloro-3-indolyl-β-D-

galactopyranoside), a clear soluble substrate, which hydrolyses to an indolyl, that then oxidises to an indoxyl and this self couples to form an insoluble idigo-blue and this allows for the localisation of transfected cells (figure 2.5.2.1). This method was adapted from the one in Methods in Molecular Biology (McGreggor 1991).

The culture media was removed from the Hec-1A cells and washed 3 times with PBS. Fix solution (3 ml/well) was added to the cells and incubated for 5 minutes at room temperature. The cells were washed with PBS prior to the addition of X-gal histochemical reaction mixture (3 ml/well) and incubated at 37 °C until the development of blue colour could be confirmed under the microscope. The cells were washed with PBS and the cells photographed (figure 2.5.2.1). All solutions are detailed within appendix II.

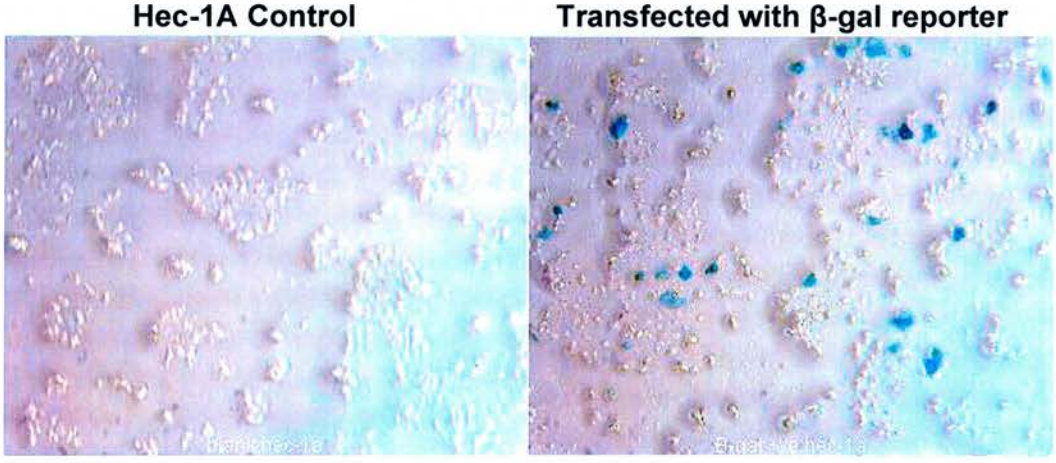


Figure 2.5.2.1 Images of Hec-1A cells transfected with the β-gal reporter construct and stained for β-galactosidase activity.

2.5.3 pCAT transfection; elafin promoter

Hec-1A cells were transiently transfected with the constructs in figure 2.5.3.1. The transfection conditions were optimised using the β-gal reporter construct (section 2.5.2). The cells were plated at 70% confluence into 6 well culture plates. The transefection complex was optimised to the following: 4 µl Fugene HD transfection reagent, 1 µg/ml plasmid DNA and 100 µl of Opti-MEM culture medium. The mixture was added to the cells in a drop-wise fashion and the plates were gently swirled to ensure an even distribution. The cells were incubated with the complex for 12 hours prior to a change in media and the addition of treatments as detailed in chapter 8.

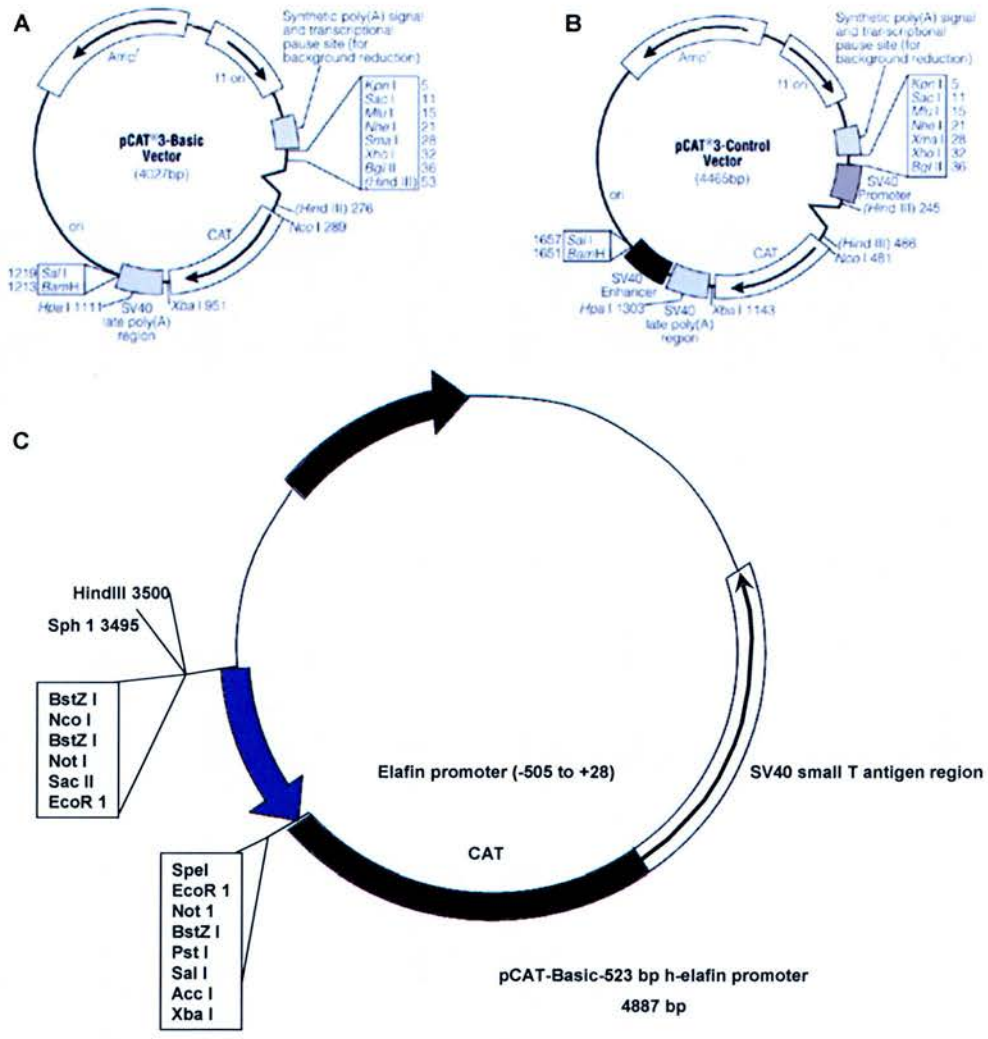


Figure 2.5.3.1 The vector maps used in the pCAT reporter assays. (A) The basic vector lacks a promoter and therefore will not express the CAT enzyme and serves as a negative control. (B) The control vector contains an SV40 promoter element which will drive the expression of the CAT enzyme and serves as a positive control for the experiments. (C) The basic vector with the SV40 promoter element replaced with the elafin promoter sequence (blue).

2.6 Immunohistochemistry

Immunohistochemistry was performed in order to detect SLPI and Elafin proteins in fixed paraffin embedded tissue biopsies (5 μm). The protocols were optimised and the appropriate conditions for maximum immunostaining were determined. The antibodies used were obtained from a variety of sources and are detailed were relevant.

2.6.1 Elafin

Tissue sections were dewaxed in xylene for 10 minutes and then rehydrated in descending grades of alcohol. Subsequent to washing with PBS, a microwave antigen retrieval step was performed (antigen retrieval solution; Vector). Non-specific endogenous peroxidase activity was blocked with 2% hydrogen peroxide (Sigma Aldrich) in distilled water for 10 minutes at room temperature. Avidin-biotin and protein blocks were applied to the tissue sections for 20 minutes each at room temperature with alternate 5 minutes PBS washes. Sections were then incubated overnight at 4 °C with rabbit anti-elafin polyclonal antibody (Sallenave, Shulmann et al. 1994) which was diluted at 1:700 in diluent (Dako antibody diluent). In negative control sections the primary antibody was substituted with an approximately equivalent Ig concentration of rabbit IgG (RIgG; Vector). Sections were incubated with biotinylated goat anti-rabbit (Vector) and were then subjected to an avidin-biotin peroxidase detection system (Vector; both were incubated for 30 minutes at room temperature). The peroxidase substrate, diaminobenzidine (Dakocytomation), was used to identify positive staining. Sections were counterstained with Harris's

haematoxylin (Pioneer research chemicals), dehydrated in ascending grades of alcohol, and mounted from xylene in Pertex (Cell path).

2.6.2 SLPI

SLPI immunohistochemistry was carried out on formalin fixed tissue obtained from the endometrium, decidua and the fallopian tube. The use of SLPI immunohistochemistry has been previously described (King, Fleming et al. 2002). The method used was identical to the elafin immunohistochemistry protocol, with the following exceptions. The primary antibody was mouse anti-SLPI (Hycult Biotechnology) diluted at 1:20 in diluent, negative controls were incubated with an equimolar concentration of mouse IgG (MIgG), and the secondary antibody was biotinylated horse antimouse Ig (Vector).

2.7 Haematoxylin and Eosin Staining

Tissue sections were dewaxed in xylene for 10 minutes and then rehydrated in descending grades of alcohol. The slides were then placed in Harris's haematoxylin (Pioneer research chemicals) for 5 minutes and washed in tap water. The sections were then placed in 1% acid alcohol solution for a few seconds and washed in tap water. The sections were placed in eosin solution for 5 minutes and washed in tap water. The sections were dehydrated in ascending grades of alcohol, and mounted from xylene in Pertex (Cell path).

2.8 Statistical Analysis

When the numerical variables were normally distributed the data was determined by Student's t-test. When results did not conform to normal distribution, non-parametric statistical analysis was performed. Fisher's protected least significant difference (PLSD) was used to assign individual differences.

Statistical significance was assigned to a p value of <0.05.

Chapter 3:

Mediators of Natural Antimicrobial Expression in the Cultured Cell line Hec-1A

3.1 Introduction

The female reproductive tract needs to be free of infection in order for there to be successful conception, implantation and pregnancy. The presence of an effective immune response is therefore essential in order to prevent deleterious infection or inflammation. It is the function of the innate immune system primarily to fulfil this role, by means of mucosal secretions and mechanical barriers. The natural antimicrobials function as part of the innate protection of the female reproductive tract, some are constitutively expressed whilst other respond to specific pathogens or stimulus.

It has been shown that the expression of natural antimicrobials can be upregulated in culture experiments with the addition of mimics of infection, such as pro-inflammatory cytokines TNFα and IL-1β (Sallenave, Shulmann et al. 1994; Diamond, Russell et al. 1996; Russell, Diamond et al. 1996; King, Fleming et al. 2002; Tsutsumi-Ishii and Nagaoka 2003; Pivarcsi, Nagy et al. 2005). These cytokines are expressed by the innate immune system in response to infection or injury and serve as the initial mechanism of alert. Investigations into the effects of specific pathogens have also been undertaken with the use of inactive cell surface proteins that are recognised by the immune system. These are described as being pathogen associated molecular patterns (PAMPs), and include lipopolysaccharide (LPS), lipoteichoic acid (LTA) and Poly I:C; representative of G-ve, G+ve bacteria and viruses, respectively (King, Fleming et al. 2002; Tsutsumi-Ishii and Nagaoka 2003; Pivarcsi, Nagy et al. 2005). The immune response to the recognition of pathogens is mediated via the Toll-like receptors (TLR). Members of the TLR

family have been shown to have specificity to different pathogens and to modulate the expression of cytokines and natural antimicrobials in response (Pasare and Medzhitov 2005).

Thus, the treatment of cell lines or primary tissue explants has allowed the study of natural antimicrobial expression. It has been shown that natural antimicrobials exhibit differential expression in response to stimuli, and natural antimicrobials respond to specific pathogens. Some antimicrobials such as hBD1 and SLPI have been shown to be endogenously expressed (Bensch, Raida et al. 1995; Williams, Brown et al. 2006). It has also been demonstrated that the differential expression of these proteins is subject to modulation by the steroid hormone levels in the female reproductive tract, as observed during the menstrual cycle (Fleming, King et al. 2003).

In this thesis, the use of the endometrial epithelial cell line Hec-1A (Kuramoto, Tamura et al. 1972) was used to investigate further the role of inflammatory mediators and steroid hormones in the expression of natural antimicrobials. The Hec-1A cell line was derived from an endometrial adenocarcinoma of glandular origin (Kuramoto, Tamura et al. 1972). Hec-1A cells have been utilised as a model for the function of endometrial glandular epithelium (Fahey and Wira 2002), and are considered to be a good model for the functional analysis of endometrial epithelia.

The aim of this study was to investigate the expression of natural antimicrobials by the Hec-1A cell line and how this might be related to the role of these proteins in the

endometrium. The data are presented in two sections; with part A, examining the effects of mimics of infection (LTA and LPS) and part B investigating the role of the steroid hormones in addition to inflammatory mediators (TNFα and IL-1β).

Chapter 3 Part A:

Temporal expression of natural antimicrobials in an endometrial epithelial cell line in response to inflammatory mimics.

3.2 Part A: Temporal expression of natural antimicrobials in an endometrial epithelial cell line in response to inflammatory mimics.

Introduction

The presence of natural antimicrobials within the female reproductive tract has been described previously (Svinarich, Wolf et al. 1997; Quayle, Porter et al. 1998; Valore, Park et al. 1998; Quayle 2002; King, Critchley et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003; Buhimschi, Jabr et al. 2004; Pivarcsi, Nagy et al. 2005). Inflammatory cytokines and other inflammatory mediators have also been shown to up-regulate the mRNA expression of NAPs in the female reproductive tract and in other systems such as the lung and the gut (Diamond, Russell et al. 1996; Jin, Nathan et al. 1998; Krisanaprakornkit, Kimball et al. 2000). The female reproductive tract has been demonstrated to yield differences in the endogenous levels of inflammatory mediators such as the cytokines IL-8, IL-1β and TNFα. These levels are variable in response to the changes associated with the menstrual cycle. The effect of time upon the expression of natural antimicrobial has not been examined previously.

Hence, this study aimed to investigate the effect of time upon the expression of natural antimicrobials in a human endometrial epithelial cell line. This was motivated by inconsistencies observed in previous experiments involving the treatment of cultured cell lines with inflammatory mimics and mediators of inflammation. It became apparent that the temporal expression of natural antimicrobials may be subject to time. Primarily these experiments were undertaken with the aim of optimising conditions in order to allow for further study. The effect

of the inflammatory cytokines TNFα, IL-1β; and mimics of infection LPS and LTA upon antimicrobial expression in the cultured cell line Hec-1A was investigated. In addition the effect of the cytokine TGFβ-1 was also examined due to some suggestions within the literature that this cytokine may have an anti-inflammatory role.

3.3 Materials and Methods

3.3.1 Cell culture

The human endometrial epithelial cell line Hec-1A (human endometrial cancer-one) (Kuramoto, Tamura et al. 1972), were cultured as described in 2.2.3.

The treatments used for this chapter are detailed in the table below (Table 3.3.1.1).

Treatment	Concentration used
Control	n/a
IL-1β	5 ng/ml
TNFα	5 µg/ml
TGFβ-1	10 ng/ml
LPS	1 µg/ml
LTA	1 µg/ml

Table 3.3.1.1 Details of treatment of Hec-1A endometrial cell line. Supplier information is contained within appendix I.

The treatments were added to the cells at time 0, and the reaction stopped and processed at each of the relevant time points (2 – 24 hr) as detailed within the results section An untreated control was included for each of the time points and this was subsequently used as the comparator for the purposes of mRNA analysis.

3.3.2 RNA extraction and Q-RT-PCR

Following treatment and incubation for the relevant time point, the RNA was extracted from the cells and cDNA prepared as described in section 2.3. Elafin,

SLPI, IL-8 and hBD mRNA levels were measured in these cDNA samples by quantitative PCR (2.3). The sequence details of the primer-probe sets used are detailed in table 2.3.3.1, materials and methods.

3.3.3 Elafin and SLPI ELISA

The culture supernatants removed from the cells during the timecourse experiments were collected, sterile filtered and assayed by the relevant ELISA as detailed in section 2.4.

3.3.4 Statistics

The data presented in this chapter is illustrative and was not subjected to statistical analysis. The charts aim to demonstrate that there is a temporal expression of mRNA for the natural antimicrobials. An n=3 set of results was selected based upon the similarities in timing, however, another n=3 could easily have been selected at a different time point.

3.4 Results

3.4.1 (a) TNFα and IL-1β have differential effects over time on the expression of natural antimicrobials (elafin, SLPI, hBD1-3 and 5).

Elafin mRNA levels are increased in response to treatment with TNFα, IL-1β and TNFα + IL-1β (figure 3.4.1.1). The addition of each of these treatments results in different expression patterns relative to time. TNFα (green) alone mediates a lower and more gradual increase in elafin mRNA when compared to the other treatments. IL-1β (red) treatment demonstrates 3 peaks in mRNA expression at 6, 12 and 18 hr; each of these peaks is immediately followed by a decrease in mRNA demonstrated at the 8 hr and 14 hr time points. The addition of TNFα + IL-1β (blue) increases the level of elafin mRNA further with maximal expression observed at 8 and 12 hr.

The amount of elafin protein (figure 3.4.1.2) demonstrates an increase and accumulation over 24 hr. Protein levels increase and are maintained at the same level between 8 and 12 hr. After 14 hr there is a sequential increase in the level of elafin protein as the timecourse progresses

Study of SLPI mRNA exhibited a phasic level of expression (figure 3.4.1.3) in response to treatment with TNFα + IL-1β. Three peaks in mRNA levels are observed at 6, 10 and 14 hr. The relative change in expression of SLPI mRNA is low and this is attributed to the high endogenous levels prior to treatment. This assertion is confirmed by the level of protein observed within the untreated culture media controls (figure 3.4.1.4). SLPI protein increases and is cumulative over time in both the untreated and treated culture media.

The expression of human β-defensins 1 (3.4.1.5); 2 (3.4.1.6); 3 (3.4.1.7) and 5 (3.4.1.8), mRNA levels were measured across time in response to treatment with TNFα + IL-1β. The patterns exhibit differences relative to time and to each other.

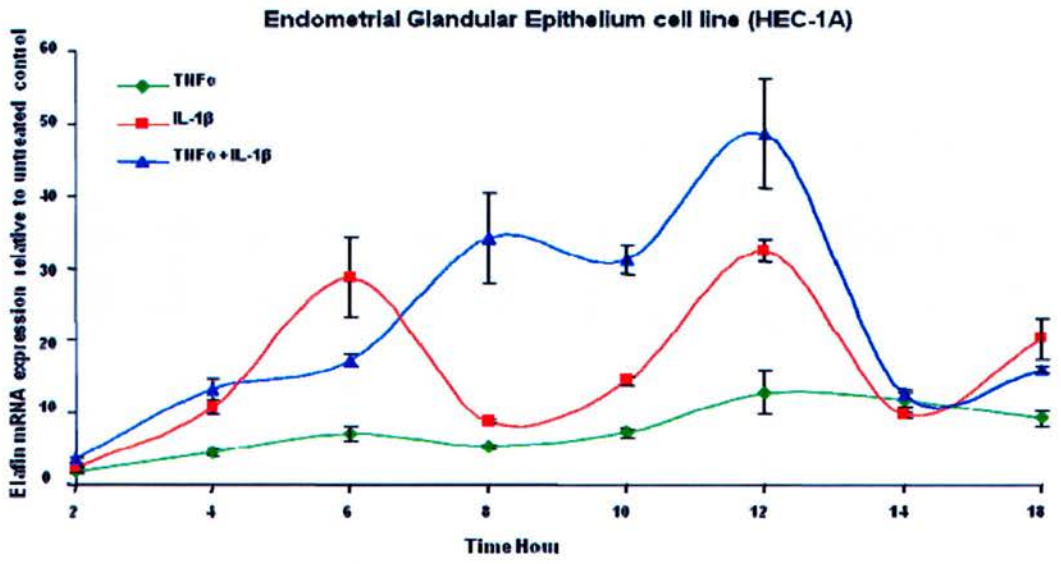


Figure 3.4.1.1. Elafin mRNA expression within the Hec-1A cells in response to treatment with TNFα (green), IL-1β (red) and TNFα + IL-1β (blue). The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

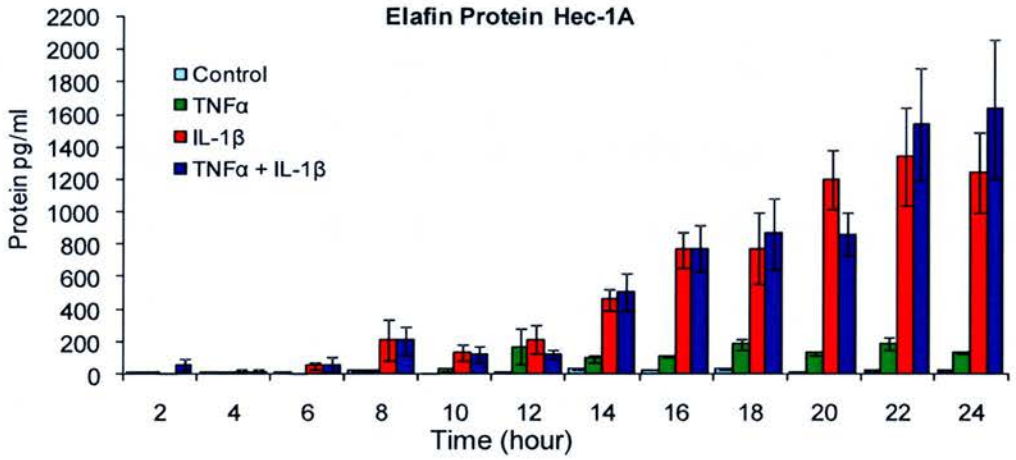


Figure 3.4.1.2. Elafin protein (pg/ml) detected within the culture media of HEC-1A cells, treated with TNFα (green), IL-1β (red), TNFα + IL-1β (blue) and untreated controls (pale blue) for 24 hr and collected at 2 hr intervals. Culture media treated with TNFα + IL-1β and IL-1β, elafin increase was significant (24hr vs 2hr; $P<0.001$). Error bars representative of ± s.e.m between experiments (n=3).

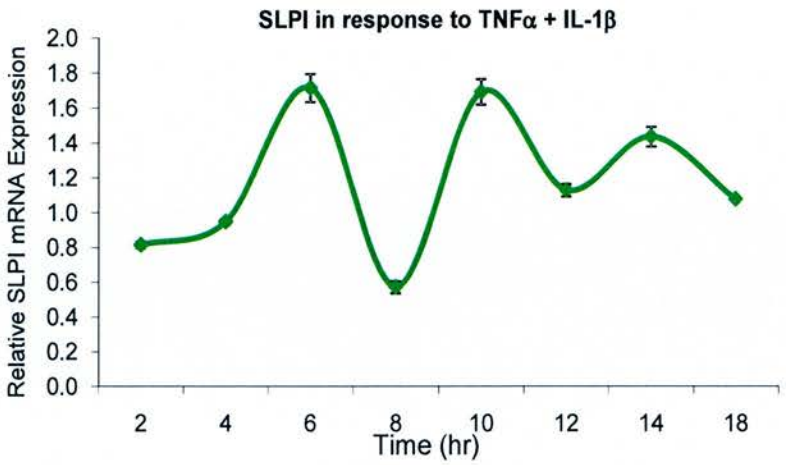


Figure 3.4.1.3. SLPI mRNA expression within the Hec-1A cells in response to treatment with TNFα + IL-1β. The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

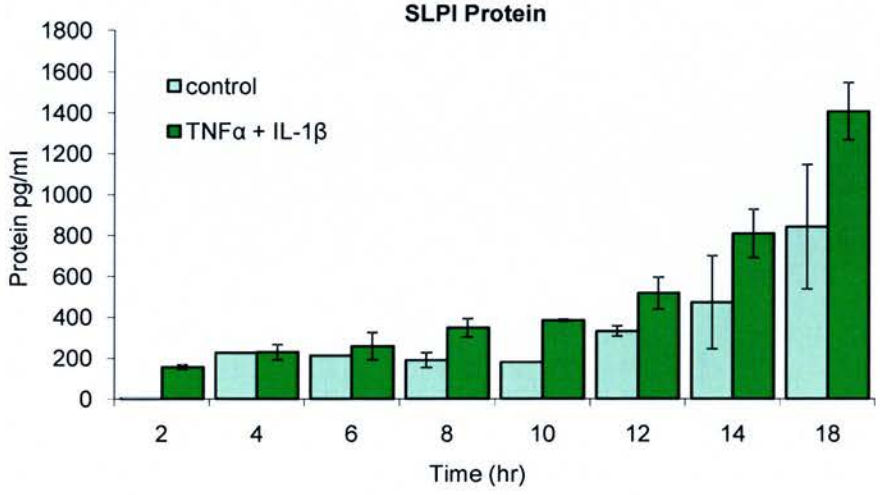


Figure 3.4.1.4. SLPI protein (pg/ml) detected within the culture media of Hec-1A cells, treated with TNFα + IL-1β (green) and untreated controls (pale green) for 14 hr collected at 2 hr intervals and at 18 hr. Error bars are representative of ± s.e.m between experiments (n=3).

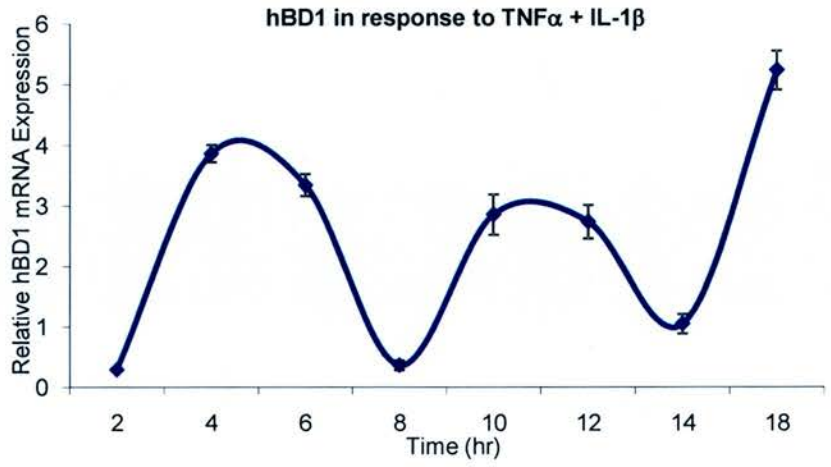


Figure 3.4.1.5. hBD1 mRNA expression within the Hec-1A cells in response to treatment with TNFα + IL-1β. The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

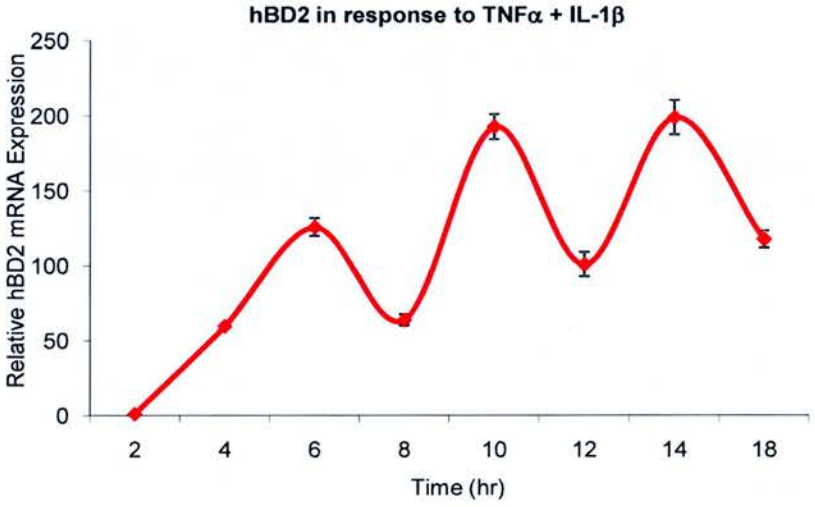


Figure 3.4.1.6. hBD2 mRNA expression within the Hec-1A cells in response to treatment with TNFα + IL-1β. The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

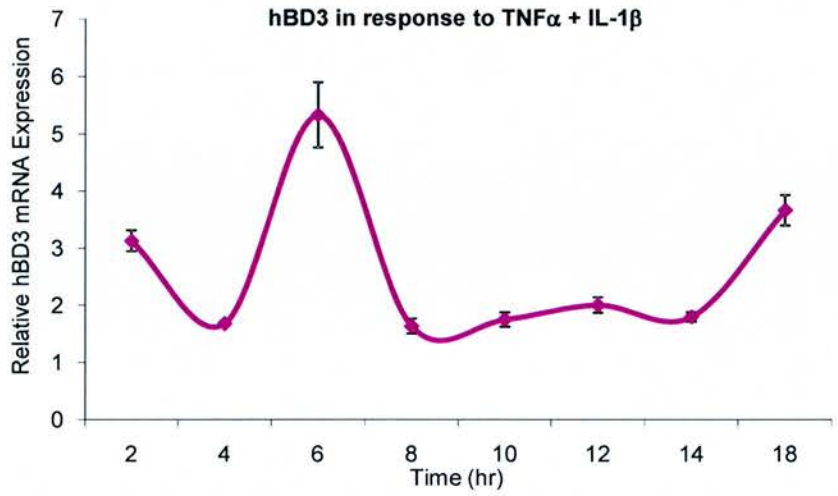


Figure 3.4.1.7. hBD3 mRNA expression within the Hec-1A cells in response to treatment with TNFα + IL-1β. The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

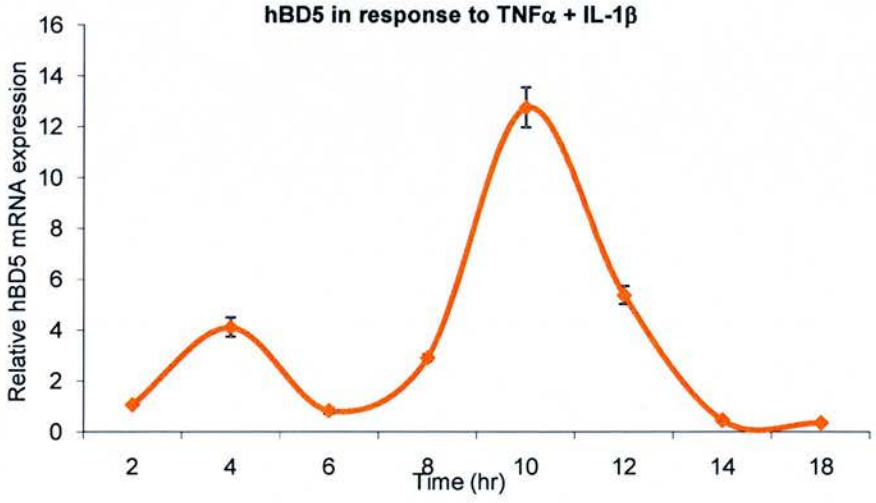


Figure 3.4.1.8. hBD5 mRNA expression within the Hec-1A cells in response to treatment with TNFα + IL-1β. The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

3.4.1 (b) The effect of inflammatory cytokines TNFα + IL-1β upon the expression of IL-6 and IL-8

In order to elucidate further the patterns of expression observed with natural antimicrobials, an investigation into the effects upon other inflammatory mediators such as the pro-inflammatory cytokine IL-8; and the anti-inflammatory cytokine, IL-6 was undertaken. These cytokines function as part of the innate immune response and respond to the same stimuli as that described for the natural antimicrobials. Thus, the response of these cytokines over time was investigated and compared to that of the natural antimicrobials.

Interleukin-6 mRNA, figure 3.4.1b (A), is increased in response to treatment with TNFα, IL-1β and TNFα + IL-1β. Messenger RNA expression is maximal for each of the conditions at the 6 hr time point. TNFα and IL-β elicit a short response with a decrease in IL-6 mRNA being noted after 6 hr. This decline in expression was observed to continue for the remainder of the examined time, with TNFα induced a slight increase 10 – 12 hr. The greatest (approx. 30) relative increase in IL-6 mRNA resulted from the addition of TNFα + IL-1β and at 6 – 8 hr, a gradual decrease is evident after 10 hr, and from 12 hr until cessation of experiment (18 hr) a relative difference of 4 of IL-6 mRNA is consistent and represents the levels observed at the beginning of the timecourse at 2-4 hr.

Interleukin-8 mRNA (B) is maximal (226 relative expression) and most rapid (4 hr) in response to TNFα + IL-1β (blue) and is immediately followed by a decrease (64

relative difference) at 6 hr. At 8 hr IL-8 increases to that observed at 2 hr (1st time point) of 140 relative expression; before gradually decreasing from 10 – 18 hr.

Interleukin-1β brings about a 140 relative increase in IL-8 mRNA expression at 2 hr and this is maintained at 4 hr. Maximal IL-8 expression in response to IL-1β is noted after 6 hr (200). There then follows a rapid decrease (18) at 8 hr, with a subsequent increase to 40 at 10 hr, which is then maintained until the end of the experiment. TNFα increases IL-8 mRNA 40 at 2 hr with a maximal increase observed at 6 hr followed by a decrease to below 30 which gradually declines to 5 at 18 hr

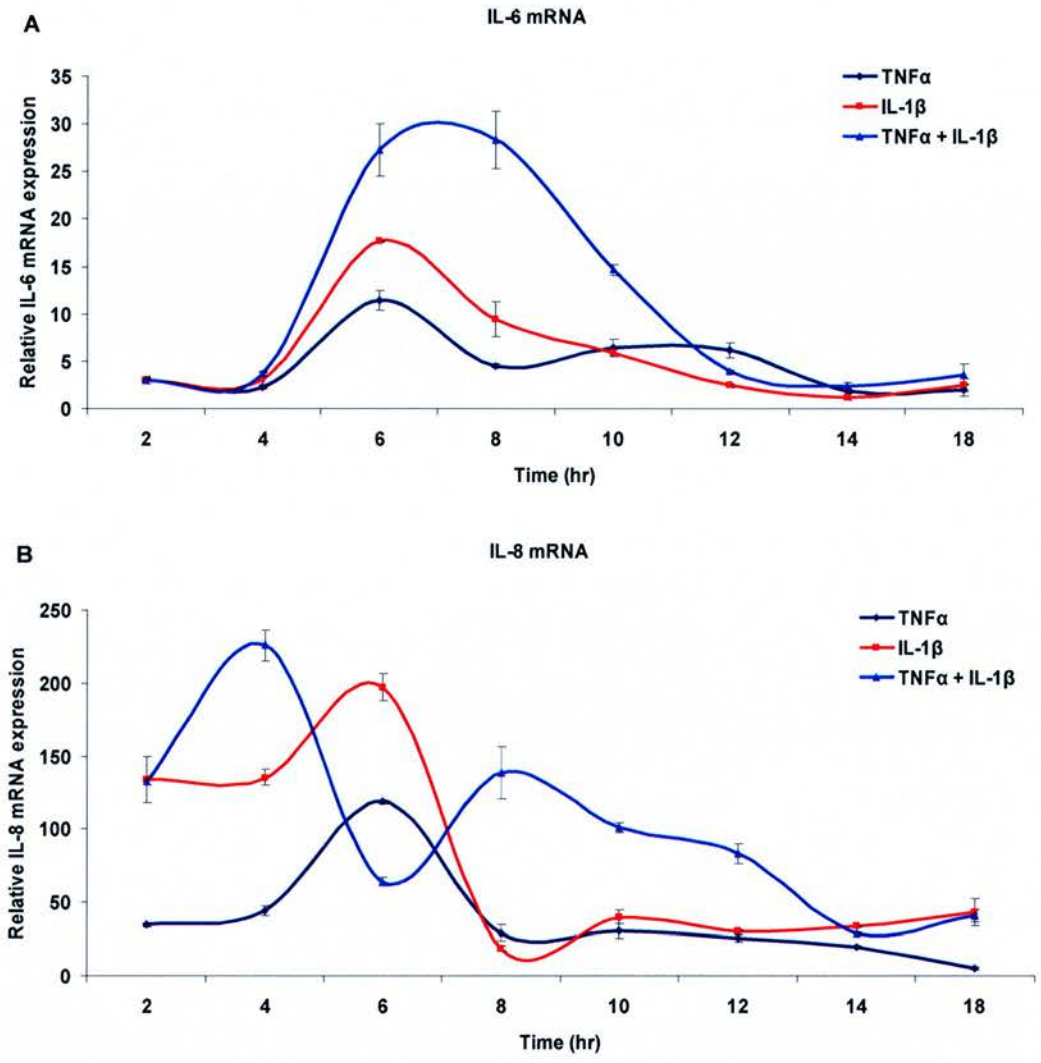


Figure 3.4.1 (b) Interleukin-6 (A) and IL-8 (B) mRNA expression within the Hec-1A cells in response to treatment with TNFα + IL-1β (note the different scales for the y axes). The samples were taken at 2 hourly intervals for 18 hr. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m (n=3).

3.4.2 Reciprocal patterns of elafin and SLPI mRNA in response to treatment with TNFα + IL-1β in HEC-1A cells.

SLPI and elafin mRNA expression in response to time was observed to demonstrate a reciprocal pattern. The relative values for each protein was plotted onto the same chart (figure 3.4.2.1). It appears that the drop in elafin mRNA coincides with an increase in the level of SLPI mRNA and vice versa. This is evident at 6, 8, 10, 12 and 14 hr. The expression of SLPI is endogenous and present at high levels basally, and thus demonstrates a smaller change. Whilst elafin expression is in response to stimulus and therefore exhibits a higher 'relative change' over an untreated control. The mRNA of the respective proteins is presented on the same graph with two axes in order for relative changes to be compared directly.

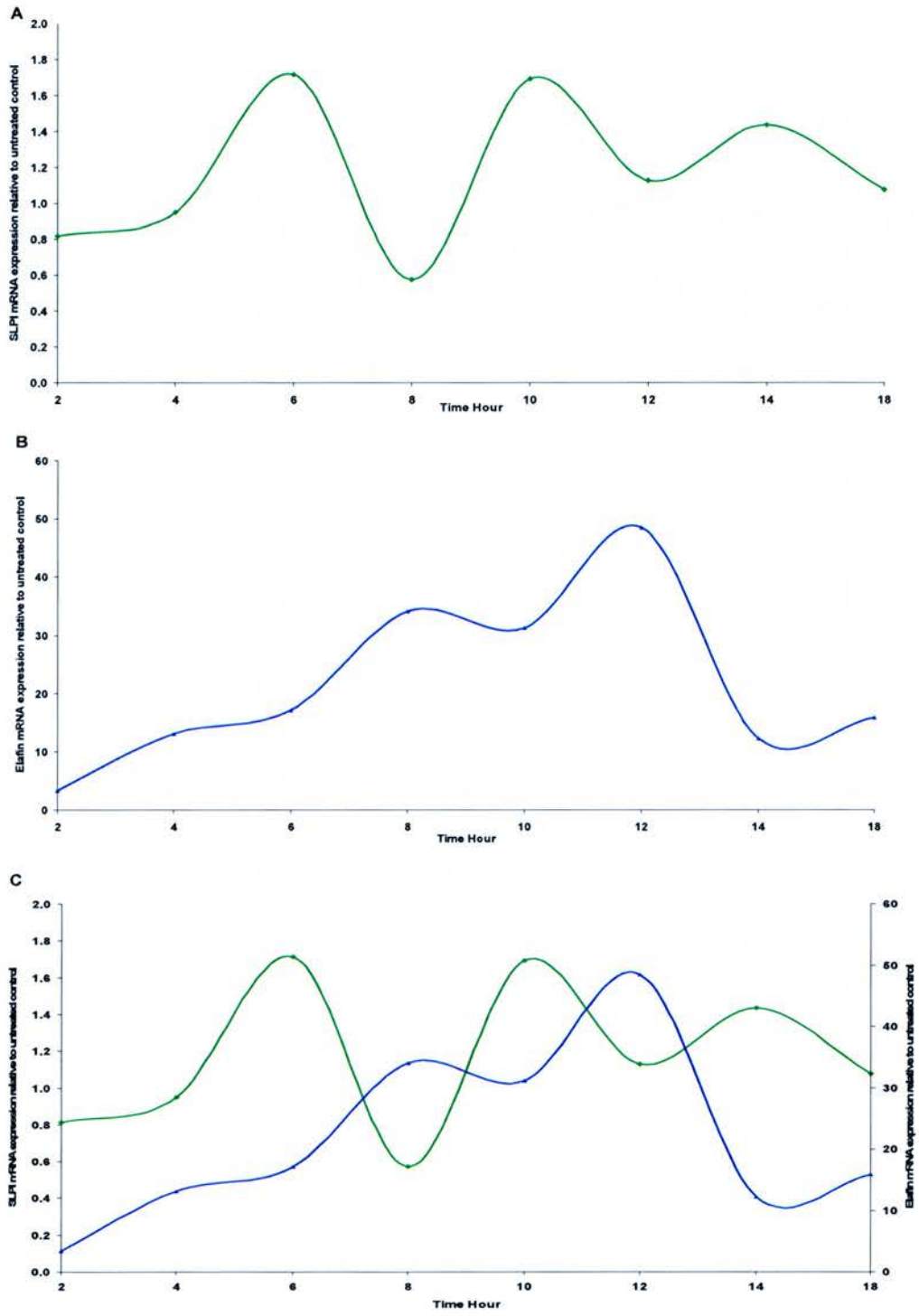


Figure 3.4.2.1 Messenger RNA expression over time, SLPI (A), elafin (B) and both (C). The axis on the left is representative of SLPI (green), whilst that on the right hand side is relevant to elafin (blue). Note the difference in the scale for elafin and SLPI.

3.4.3 The effect of TGFβ-1 on natural antimicrobial mRNA expression of cells treated with inflammatory mediators.

The results of the timecourse experiments demonstrated the upregulation of natural antimicrobials in response to treatment with mimics of infection. However, it was also apparent that there was a rapid downregulation in mRNA expression following peak expression levels. This led us to question the possibility of interactions with anti-inflammatory mediators. It has been suggested in the literature that TGFβ-1 may have such a role during an inflammatory event (Fadok, Bratton et al. 1998). Therefore it was decided to examine the effects of TGFβ-1 on the expression of natural antimicrobials in epithelial cells treated with mimics of infection.

The addition of TGFβ-1 to Hec-1A cells treated with IL-1β, LPS + IL-1β and LTA + IL-1β reduced the expression of elafin mRNA when compared to treatment with the inflammatory mediators alone figure 3.4.3.1. However, there appeared to be little or no effect of TGFβ-1 upon elafin mRNA when treated with either LPS or LTA. The decrease in elafin mRNA expression observed in cells treated with TGFβ-1 + IL-1β compared with IL-1β; and TGFβ-1 + IL-1β + LTA compared with IL-1β + LTA was significant ($n=4$; $P<0.001$).

Cells were also treated with the pro-inflammatory cytokines IL-1β + TNFα, with (red) and without (blue) TGFβ-1; figure 3.4.3.2. Elafin expression was shown to be significantly decreased with the addition of TGFβ-1 ($n=8$; $P<0.05$)

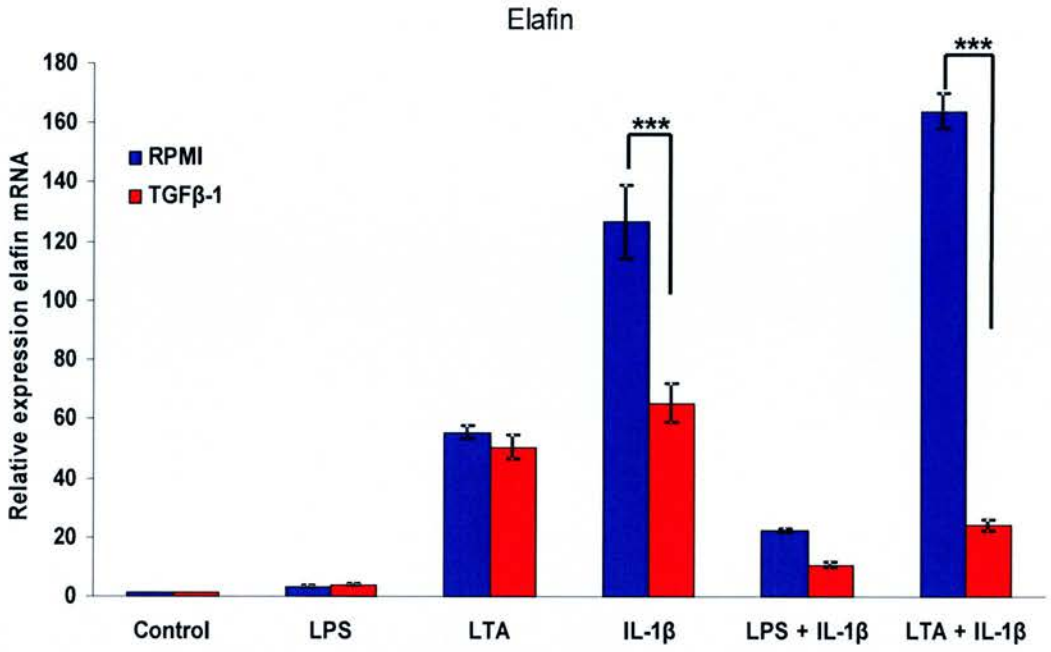


Figure 3.4.3.1 Elafin mRNA expression in Hec-1A cells in response to treatment with mimics of infection; LPS, LTA, IL-1β, LPS + IL-1β and LTA + IL-1β (blue); the addition of TGFβ-1 (red). The data are presented as relative to a control (untreated HEC-1A cells for each time point); given a nominal value of 1, mean ± s.e.m. Each experiment (n=4) was performed at 4, 8 and 12 hr; the 3 time points were averaged and served to allow for variances in response to time.

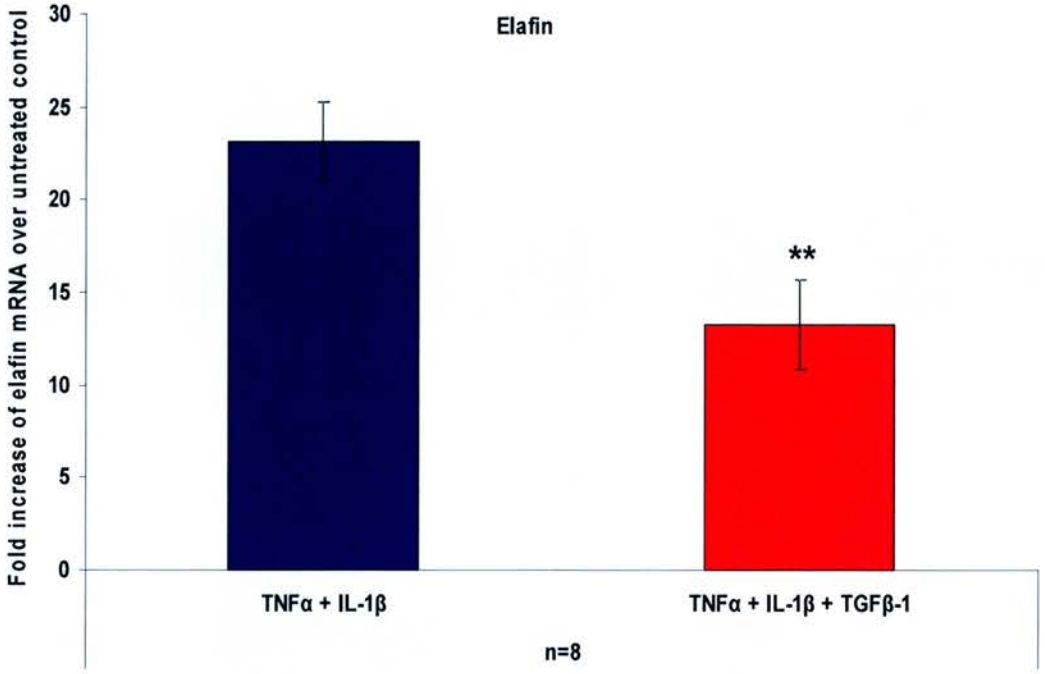


Figure 3.4.3.2 Elafin mRNA expression in Hec-1A cells in response to treatment with pro-inflammatory cytokines TNFα + IL-1β (blue); the addition of TGFβ-1 (red). The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ±s.e.m. Each experiment (n=8) was performed at 4, 8 and 12 hr; the 3 time points were averaged and served to allow for variances in response to time.

Summary of results:

1. Natural antimicrobial expression is differential across time.
2. Natural antimicrobial expression is phasic and distinct for each protein.
3. Messenger RNA expression of elafin and SLPI appears to be reciprocal.
4. TGFβ-1 inhibits the inflammatory expression of elafin mRNA

3.5 Discussion

Mucosal surfaces act as a barrier to infection and the epithelial cells of these surfaces are the main components of this barrier. Epithelial cells are known to express a number of inflammatory mediators in order to ensure an adequate immune response. In the event of an infection, epithelial cells express pro-inflammatory cytokines such as IL-8, IL-1β and TNFα, which function centrally to the innate immune response. These cytokines have also been demonstrated to have a role in the expression of natural antimicrobials, with the treatment of cell lines such as those of the lung (Sallenave, Shulmann et al. 1994; Tsutsumi-Ishii and Nagaoka 2003); a breast cancer cell line (King, Morgan et al. 2003) and a vaginal epithelial cell line (Pivarcsi, Nagy et al. 2005). In the endometrium the cell lines MFE-296 (Hackenberg, Beck et al. 1994) and HES (Desai, Kennard et al. 1994) have similary been shown to be responsive to pro-inflammatory cytokines (King, Fleming et al. 2002) causing an increase in natural antimicrobial expression. It has been shown in this thesis that the endometrial cell line Hec-1A expresses natural animicrobials both constitutively and through stimulation with inflammatory cytokines. Although, a rapid response to infection is necessary it is also important that such a reaction is short acting and downregulated in order to prevent too much inflammation which can be damaging to the host. Thus, as part of the innate response there are also anti-inflammatory mediators which serve to reduce the expression of pro-inflammatory mediators. There have been many anti-inflammatory mediators described in the literature including the cytokine TGFβ-1, which has been shown to inhibit the expression of SLPI (Jaumann, Elssner et al. 2000).

In this chapter the expression of natural antimicrobials by Hec-1A cells has been demonstrated to be both temporal and differential in relation to time. The expression pattern of the β-defensins, SLPI and elafin were found to be different, with many showing considerable overlap, for example across the 2-14 & 18 hr period. The expression patterns of the natural antimicrobials are summarised, demonstrating the times of maximal expression in relation to one another (figure 3.5.1). There is maximal expression of different antimicrobials at different times with some being co-expressed, such as hBD1 and hBD3 at 18 hr. It has also been suggested within the literature that the actions of natural antimicrobials and/or expression may also be synergistic or additive in nature (Nagaoka, Hirota et al. 2000; Singh, Tack et al. 2000; Yanagi, Ashitani et al. 2007). Perhaps the overlapping nature of mRNA expression across time is also suggestive of such combined actions.

The schematic summary of the peaks and troughs in mRNA expression of natural antimicrobials (figure 3.5.1) demonstrates that there is broad coverage across time with at least one antimicrobial (mRNA) being expressed at each time point. It was also consistently observed that maximal expression of mRNA was obtained in response to treatment with both IL-1β and TNFα, suggesting a synergistic action of these cytokines upon the expression of natural antimicrobials (King, Fleming et al. 2002). This interaction between IL-1β and TNFα has also been previously described in other systems (Moore, Lahiri et al. 2001).

The analysis of elafin and SLPI protein levels showed an accumulation in concentration, however, it is of note that there are high levels of SLPI protein in the

untreated cell culture media – confirming the observations of constitutive expression. However, questions arise surrounding the accumulative increase of SLPI protein observed in untreated samples – timed constitutive expression; stress of serum removal? Elafin protein was detected at low levels after 8 hr, which may be an indication of the time required from the time of mRNA production to translation. However, it is difficult to make this conclusion with the current data and presently the time between elafin mRNA expression and the production of protein is not known. It may be relevant that the fall in the level of elafin mRNA (fig 3.4.1.1) expression appears to be mirrored in a slightly lowered level of protein (fig 3.4.1.2) at 10 hr.

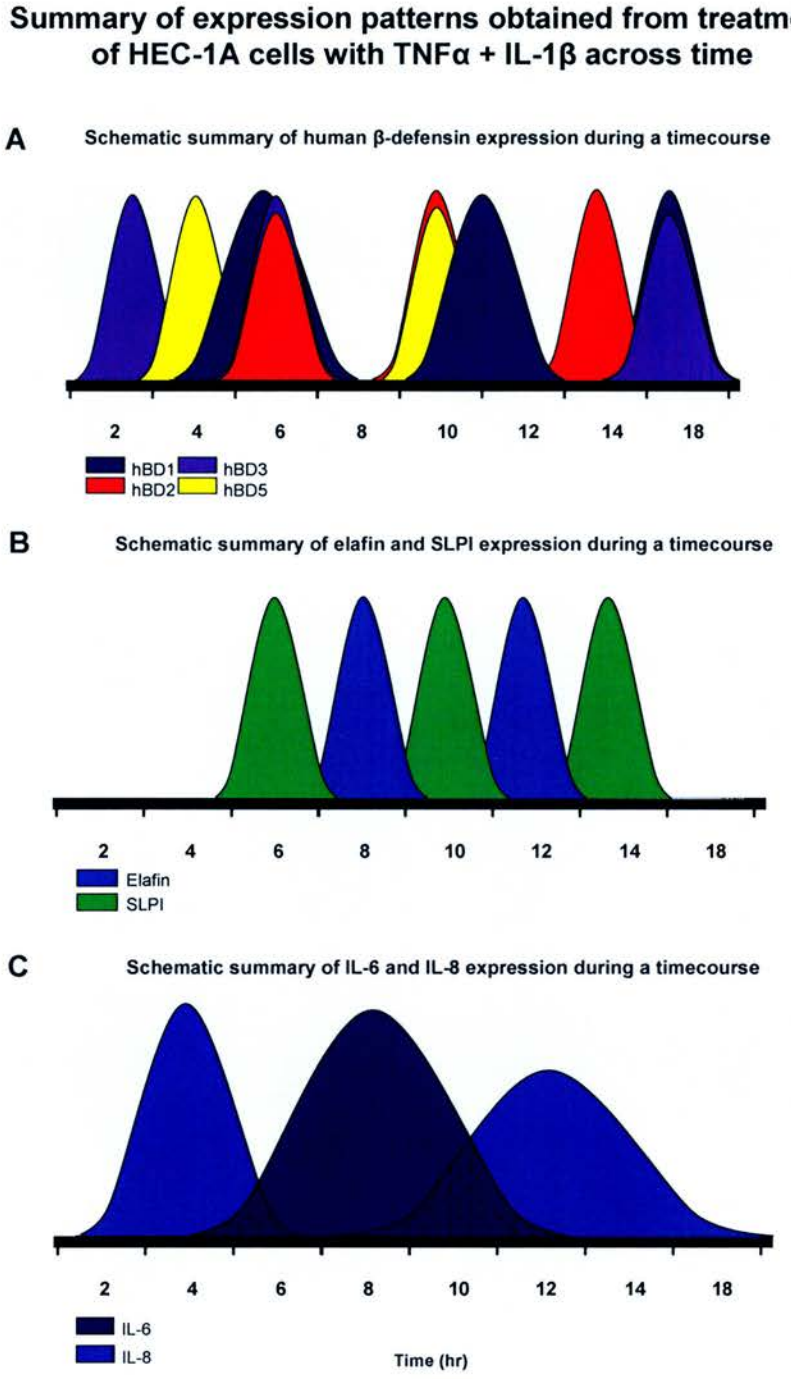


Figure 3.5.1 Schematic summary of the maximal expression of defensins 1-3 & 5 (A); SLPI and Elafin (B) and pro-inflammatory cytokines (C) in relation to time, based upon the mRNA data presented in this chapter.

It has been suggested that many innate immune mediators are produced as precursors and stored, and that in the event of infection or injury are spliced or cleaved into their active form (Raj and Dentino 2002; Guyot, Zani et al. 2005; Weeks, Tanabe et al. 2006). It is known and was discussed in chapter 1 that most NAPs have pre-active forms, and currently, there is much speculation into the possible mediators or enzymes involved in this transformation.

The examination of mRNA of the cytokines IL-6 and IL-8 was also undertaken, this was to enable a comparison to be made with other innate immune mediators. The cytokines also demonstrated temporal mRNA expression in response to treatment with inflammatory mediators; however, this was more gradual. This suggested that the rapid up-regulation and down-regulation in natural antimicrobial mRNA expression may be unique to them, as opposed to a common response exhibited by all innate immune respondents. There is also the possibility of a feedback mechanism whereby the timed response of one molecule brings about the upregulation of another. Thus, the upregulation of inflammatory cytokines such as IL-8 may be responsible for the sequential increase in NAP mRNA as observed herein. It would be useful to undertake experiments whereby the relevant cytokines are antagonised or blocked, and the resultant expression patterns of NAPs examined. It also has to be considered that in this artificial set-up there is no adaptive immune response available. The consequence is that many of these proteins function as short acting alarm signals and as chemotactic agents for the adaptive mediators, which are thought to 'take over' and resolve inflammation etc. Perhaps, this lack of adaptive response gives rise to re-signalling events as a possible explanation for the phasic

and transient expression of NAP mRNA. The upregulation of natural antimicrobial mRNA may serve as signalling mechanism for the action of other molecules. A consequence of this may be the downregulation of the NAPs, in the form of a self-regulatory mechanism in the prevention of sepsis or damage resulting from an over zealous immune response.

The examination of the mRNA expression patterns of SLPI and elafin demonstrated a reciprocal relationship. In order to show this relationship the relative mRNA data for the respective proteins was plotted on two axes (figure 3.4.2.1). The suggestion of a relationship or association between these two molecules has not previously been demonstrated. A previous observation on the expression of these proteins in the endometrium has shown them to be expressed at different times during the menstrual cycle (King, Critchley et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003). The possible association between these two proteins is examined further in the later chapters of this thesis (chapters 6-8).

There are a number of molecules that have been reported to have an anti-inflammatory mode of action within the immune response. TGFβ-1 has been shown to inhibit the expression of SLPI mRNA in human bronchial epithelial cells (Jaumann, Elssner et al. 2000). The effect of the cytokine TGFβ-1 was investigated as part of this study in order to examine its possible role in the limitation of an inflammatory response. Here it is shown that TGFβ-1 has an inhibitory effect upon the expression of elafin mRNA (figure 3.4.3.1) and this has not previously been observed. It has been reported that TGFβ-1 is decreased during the secretory phase

of the menstrual cycle in rhesus monkeys (Ace and Okulicz 2004). Thus, it may be possible that the decrease in the level of TGFβ-1 in the secretory phase may in part contribute to the observed increase in elafin expression during the late secretory and menstrual phases of the human menstrual cycle. However, there have been no conclusive studies of cyclical changes in TGFβ-1 expression, but, an increase in the level of the isoform TGFβ-3 is observed in the late secretory phase of the human endometrium (Jones, Stoikos et al. 2006). The co-treatment of the Hec-1A endometrial epithelial cell line with TGFβ-1 in this chapter has provided some preliminary data that has shown that there is an inhibition of SLPI mRNA, confirming the observations of Jaumann et al. in bronchial epithelial cells (Jaumann, Elssner et al. 2000), figure 3.5.2.

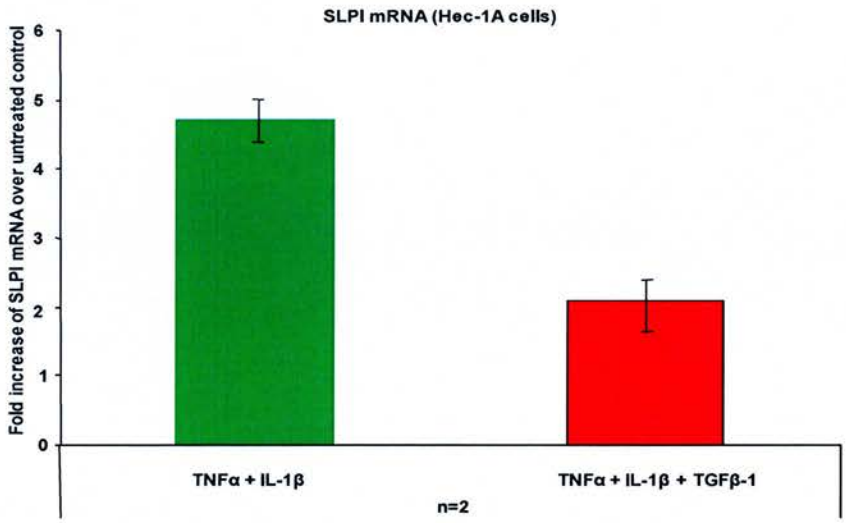


Figure 3.5.2 SLPI mRNA expression in Hec-1A cells in response to treatment with pro-inflammatory cytokines TNFα + IL-1β (green); the addition of TGFβ-1 (red). The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ±s.e.m. Each experiment (n=2) was performed at 4, 8 and 12 hr; the 3 time points were averaged and served to allow for variances in response to time

The expression of hBD4 mRNA was not detected during this study and this is consistent with the literature. This protein is not upregulated by inflammatory cytokines (De Smet and Contreras 2005). The expression of hBD4 has been observed in the testis (Yamaguchi, Nagase et al. 2002) and in the proliferative endometrium (King, Fleming et al. 2003). There have been no reports of hBD4 expression in epithelial cell lines.

In summary, it has been shown here that the expression of natural antimicrobial mRNA is both temporal and differential in relation to time. It has also been demonstrated that the expression patterns (figure 3.5.1) for the natural antimicrobials may be suggestive of combined or cooperative action. The expression patterns for SLPI and elafin appear to be reciprocal in relationship, and will be investigated further (chapters 6-8).

The cytokines IL-8 (inflammatory) and IL-6 (anti-inflammatory), exhibited a different pattern of mRNA expression than that observed for the natural antimicrobials (figure 3.5.1). Finally, it has been shown that TGFβ-1 has an inhibitory action on elafin mRNA expression and this has been previously reported for SLPI (Jaumann, Elssner et al. 2000). It is not possible to conclude whether this effect is direct or indirect based on the data presented and this issue will be subject to further investigation in this thesis (chapt 8). The data presented in this chapter is summarised in figure 3.5.3.

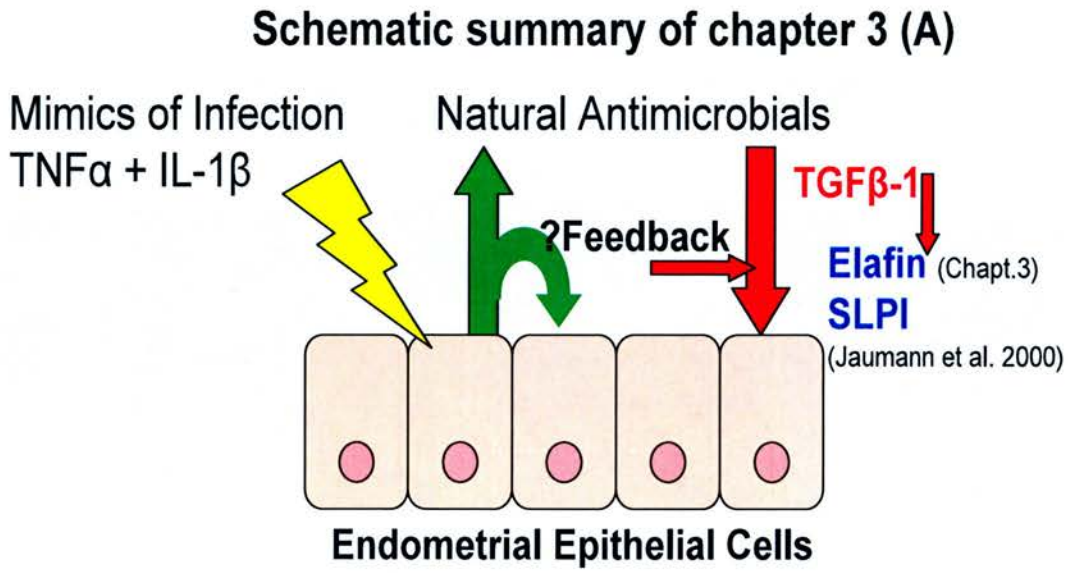


Figure 3.5.3 Mimics of infection and pro-inflammatory cytokines up-regulate the expression of NAPs in the endometrial cell line (Hec-1A). Timecourse analysis demonstrated a sequential down-regulation of NAPs. Further investigation into the possible mechanisms and factors involved in this down-regulation of expression was deemed necessary.

3.5.1 Future work

There were a number of questions that arose during the investigations undertaken within this chapter. The timecourse experiments demonstrated the rapid upregulation of natural antimicrobials. However, the observation that this upregulation was followed by downregulation gave rise to the question of what factor(s) or mechanisms could be responsible for this downregulation. As discussed previously, from the literature and the work presented herein, TGFβ-1 has been identified as an inhibitor for the expression of both SLPI and elafin mRNA. In order to fully elucidate the inhibitory nature of TGFβ-1, it would be necessary to examine the effects upon other innate immune effectors, such as the β-defensins and both pro- and anti-inflammatory cytokines. It may be that TGFβ-1 mediates its inhibitory effect indirectly through interaction with other cytokines for example if TGFβ-1 could also downregulate epithelial production of pro-inflammatory cytokines, such as IL-1β and TNFα.

Chapter 3 Part B:

The effect of steroid hormones and inflammatory mediators on the expression of natural antimicrobials in an endometrial epithelial cell line

3.6 Part B: The effect of steroid hormones and inflammatory mediators on the expression of natural antimicrobials in an endometrial epithelial cell line.

Introduction

It has been shown that natural antimicrobial expression is variable through the menstrual cycle (King, Critchley et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003). It has been further shown that this expression can be suppressed in the presence of hormonal contraceptives (Fleming, King et al. 2003). The hormonal influence over natural antimicrobial expression could be direct, for example, SLPI which has been shown to have a progesterone response element (PRE) like sequence (Velarde, Iruthayanathan et al. 2006). The effect may also be indirect as hormones are known to modulate the expression of other molecules such as pro-inflammatory cytokines which may in turn act upon natural antimicrobials (Tabibzadeh and Sun 1992; Simon, Piquette et al. 1993; Hunt, Miller et al. 1997). SLPI has previously been demonstrated to be maximal during the mid secretory phase of the menstrual cycle when circulatory progesterone levels are maximal (King, Critchley et al. 2003). SLPI was also found to be upregulated *in vitro* in the breast cancer cell line (T47D) in response to treatment with progesterone (King, Morgan et al. 2003). Elafin is upregulated during the progesterone withdrawal mediated late secretory phase and is maximally expressed during the menstrual phase when progesterone levels are low (King, Critchley et al. 2003). Human β-defensin 2 has also been shown to be maximally expressed during the menstrual phase suggestive of progesterone inhibition (Fleming, King et al. 2003). Human β-defensin 1 has been reported to be constitutively expressed in other systems

(Krisanaprakornkit, Weinberg et al. 1998; O'Neil, Cole et al. 2000). In the endometrium it has been shown that hBD1 mRNA is maximal during times of high progesterone (King, Fleming et al. 2002). Previous work within our laboratory demonstrated the maximal expression of granulysin in late secretory endometrium and it has been suggested that this may originate from the uNK cells (Fleming, King et al. 2003; King, Critchley et al. 2003).

This study investigates the expression of natural antimicrobials in the endometrial epithelial cell line (Hec-1A), in response to mimics of infection and the sex steroid hormones. How this relates to antimicrobial expression in endometrium through the menstrual cycle and how this might relate to function will be considered. The expression of natural antimicrobials both in response to sex steroids and without has not previously been described for Hec-1A cells in culture.

3.7 Materials and Methods

3.7.1 Cell culture and treatments

The Hec-1A cells were cultured as described in section 3.3.1. The epithelial cells were then treated with inflammatory mediators IL-1β and TNFα, (table 3.3.1.1) with the addition of the hormonal steroid progesterone (10^{-6} M; (Kelly, King et al. 2002)) and the progesterone antagonist mifepristone (RU486; 10^{-6} M). Progesterone and RU486 are prepared in ethanol and thus ethanol was added to the relevant controls.

3.7.2 RNA extraction and Q-RT-PCR

Following treatments the cells were harvested in Tri reagent, RNA extracted and cDNA prepared as described in section 2.3.2. The level of natural antimicrobial mRNA was determined using Taqman RT-PCR (see section 2.3.3 for details of the method, the sequence details for all primer sets used are detailed in table 2.3.3.1).

3.7.3 Statistical analysis

The PCR results in this chapter were analysed by ANOVA for significant difference. Fisher's protected least significant difference (PLSD) was used to assign individual differences (PRISM). No analysis was performed on the co-culture data due to low number.

Each n=1 is equal to the mean of 3 individual time points (4, 8 and 12 hr), in order to account for the variances in expression across time and allow for comparison on the basis of treatment conditions alone.

3.8 Results

3.8.1 *In vitro* treatment of HEC-1A cells with progesterone increases elafin mRNA expression in response to inflammatory mimics.

Elafin mRNA was increased in the presence of progesterone when compared to cells treated in RPMI culture media (figure 3.8.1.1). There is a 30 relative level of increase in elafin mRNA in cells treated with IL-β and progesterone; and an 80-point increase in response to IL-β + TNFα. The data are presented as relative to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ± s.e.m. Three time points 4, 8 and 12 hr were obtained and the combined mean value equated to n=1; to allow for the fluctuations in expression observed in part A

3.8.2 Progesterone stimulates granulysin mRNA expression in response to inflammatory mimics.

Granulysin mRNA increased in the presence of progesterone and mimics of infection when compared to an untreated control (figure 3.8.2.1). At least a 15 relative increase was observed when Hec-1A cells where treated with IL-1β and progesterone (blue), when compared to non-progesterone (RPMI) IL-1β treated cells (green; undetected). However, the addition of TNFα, LPS and LTA with IL-1β reduced the level of granulysin mRNA compared with IL-1β alone. The samples treated with inflammatory cytokines in the presence of progesterone demonstrated a significantly higher level of expression over RPMI ($P<0.001$).

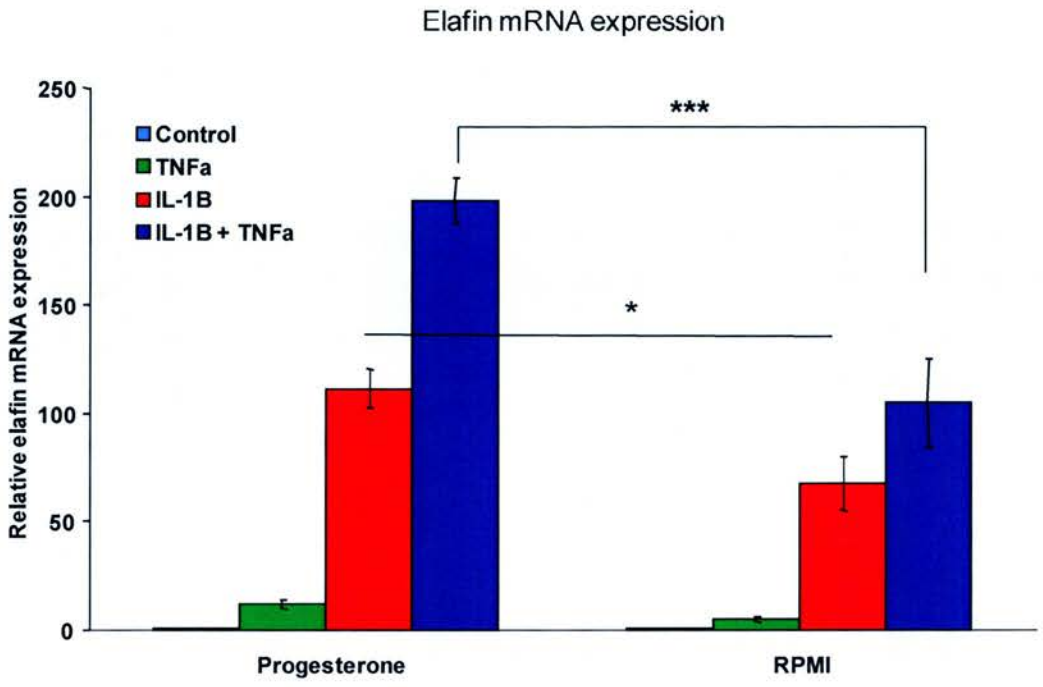


Figure 3.8.1.1 Elafin mRNA expression in Hec-1A cells treated with the pro-inflammatory cytokines TNFα, IL-1β and in combination. The treatments were undertaken with or without progesterone. The increase in elafin expression in the presence of progesterone is significantly increased for treatment with IL-1β (red; $P<0.05$) and IL-1β + TNFα (blue; $P<0.001$). Data presented as relative to an untreated control (progesterone is in an ethanol solution thus, controls were treated with an equal volume of ethanol), and represents 3 separate time points (4, 8 & 12 hr), n=5.

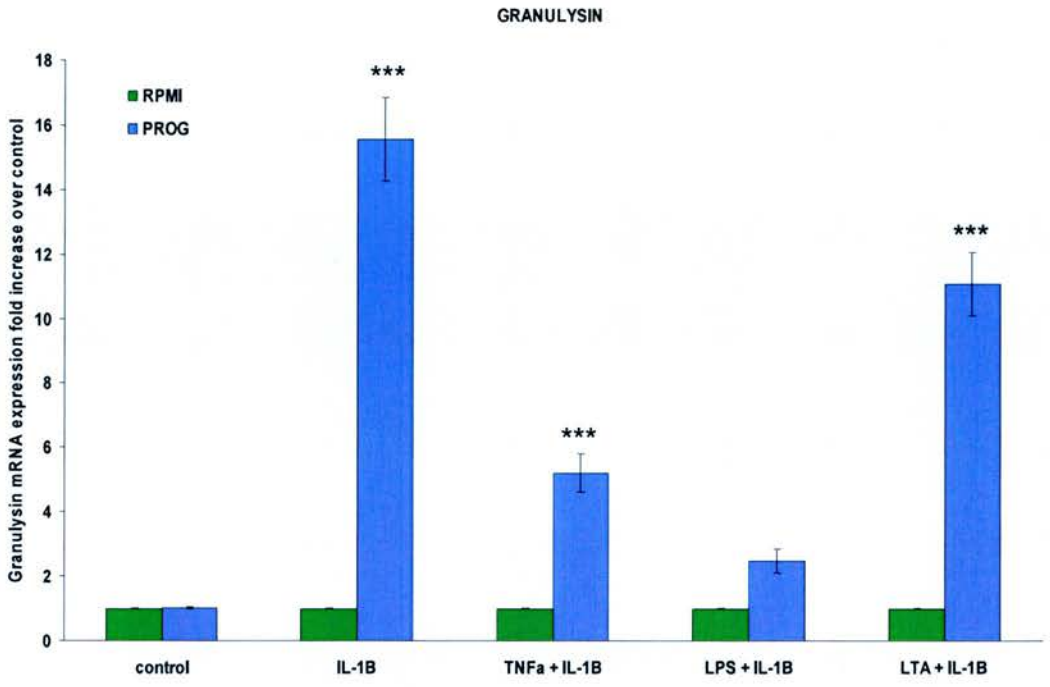


Figure 3.8.2.1 Granulysin mRNA expression in Hec-1A cells treated with IL-1β, TNFα + IL-β; LPS + IL-1β; and LTA + IL-1β. The treatments were undertaken with (blue) or without progesterone (green). Data presented as relative to an untreated control (progesterone is in an ethanol solution thus, controls were treated with an equal volume of ethanol), and represents 3 separate time points (4, 8 & 12 hr), n=4. $P<0.001$

3.8.3 The comparative effect of progesterone and progesterone antagonism with RU486 on the expression of natural antimicrobials.

The effect of the progesterone and the progesterone antagonist RU486 upon the expression of antimicrobials was examined.

SLPI mRNA (figure 3.8.3.1) was maximal with progesterone and inflammatory mediators TNFα + IL-1β; at least 4 points greater than the RPMI equivalent ($P<0.001$). The synergistic response usually observed with TNFα + IL-1β treatment is not observed in the cells treated in RPMI. Concurrent treatment with RU486 or RU486 + progesterone, and pro-inflammatory cytokines; resulted in a decrease in the SLPI mRNA when compared to RPMI (not significantly)

Elafin mRNA (figure 3.8.3.2) was observed to increase when treated with pro-inflammatory cytokines in the presence of progesterone; IL-1β prog 27 points increase over IL-1β RPMI; TNFα + IL-1β 30-point increase and there was no effect with TNFα alone. Treatment with RU486 decreased elafin mRNA and the synergism was lost when compared to progesterone treatment, however, the addition of RU486 demonstrated a 10-point increase over that observed for RPMI with IL-1β, whilst there was no change in the response to IL-1β + TNFα. Maximal elafin mRNA expression was observed in the presence of progesterone + RU486 when compared to all other treatments. The greatest increase was demonstrated with IL-1β 25-point increase over the progesterone counterpart. It was also apparent that the synergistic effect obtained from both cytokines is not maintained under these conditions, although there is a 6-point increase over progesterone alone it is 13-point lower than

the level obtained from IL-1β alone under the same treatment conditions. Both the RU486 and the RU486 + progesterone treatments give rise to this 'inhibition of synergism' phenomenon.

Human β-defensin 2 mRNA (figure 3.8.3.3) was decreased when treated with progesterone in comparison with cells treated without the presence of progesterone (RPMI). In the presence of progesterone, a 45-point decrease in expression in response to IL-1β; and a 65-point decrease in response to treatment with IL-1β + TNFα, when compared to treatment without progesterone. A further decrease was observed in samples treated in the presence of RU486 – 10-point reduction in comparison with prog samples for IL-1β and a 17-point reduction for IL-1β + TNFα. Concurrent treatment with both progesterone and RU486 gave rise to maximal hBD2 expression within these experiments. 110 IL-1β and a 152 for IL-1β + TNFα; 66 and 94

Nearly a 45 point increase over RPMI for IL-1β; and a 58-point increase for treatment with IL-1β + TNFα

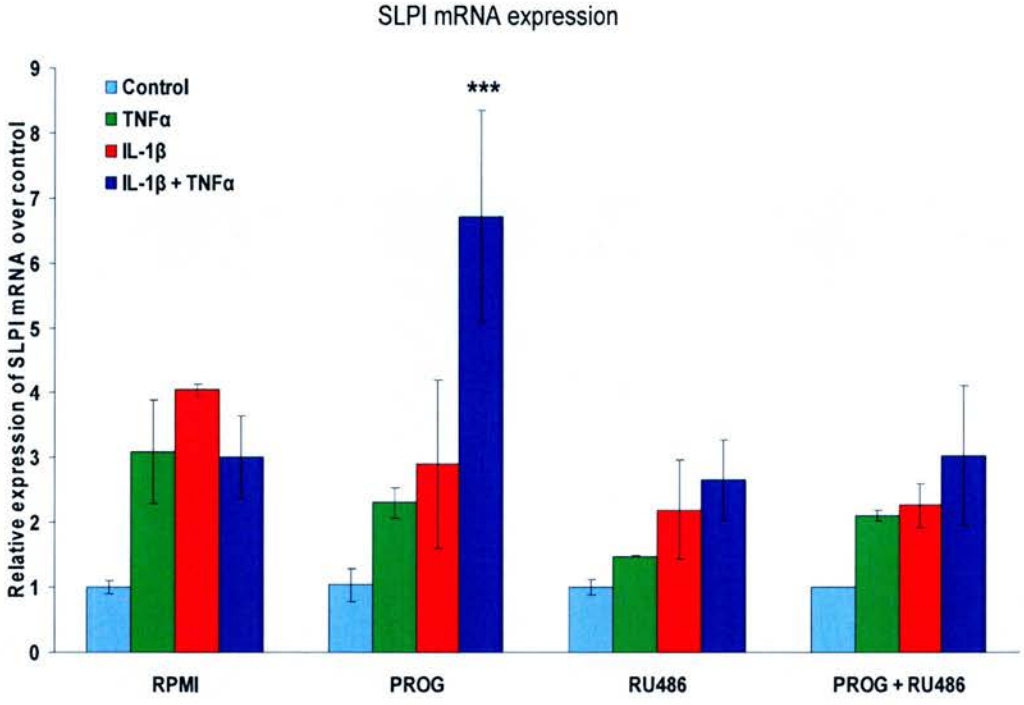


Figure 3.8.3.1 SLPI mRNA expression in Hec-1A cells treated with the pro-inflammatory cytokines TNFα, IL-1β and in combination. The treatments were undertaken with RPMI, progesterone, RU486 and progesterone + RU486. The increase in SLPI expression in the presence of progesterone is significantly increased for treatment with IL-1β + TNFα (blue; $P<0.001$). Data presented as relative to an untreated control (progesterone and RU486 are in an ethanol solution thus, controls were treated with an equal volume of ethanol) and represents 3 separate time points (4, 8 & 12 hr), n=5.

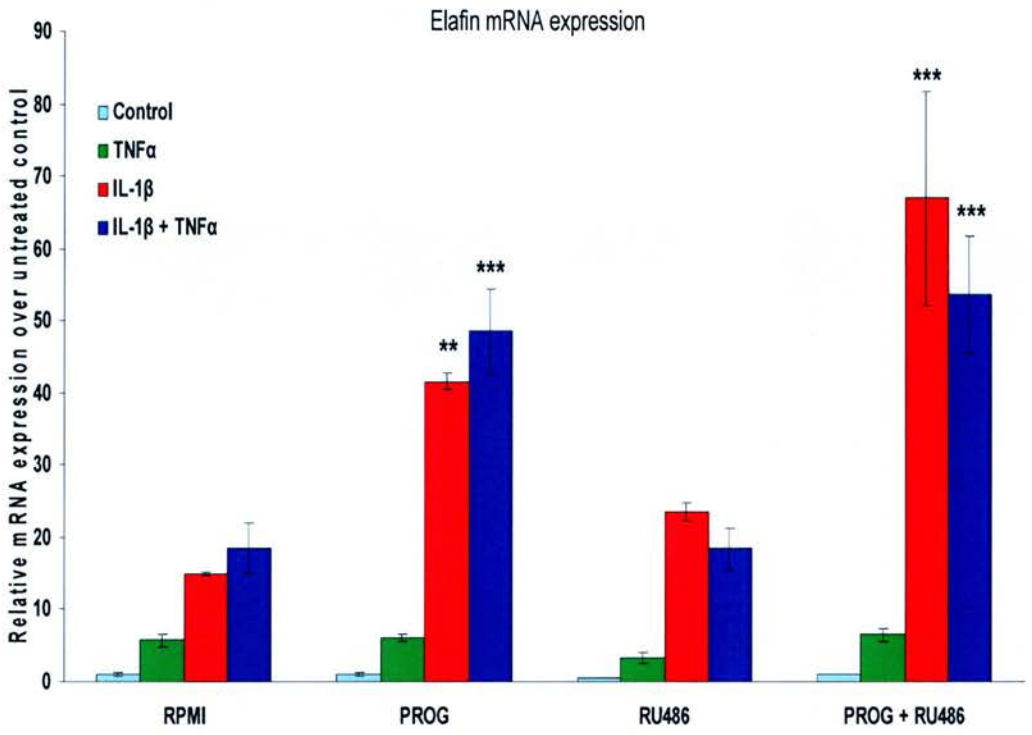


Figure 3.8.3.2 Elafin mRNA expression in Hec-1A cells treated with the pro-inflammatory cytokines TNFα, IL-1β and in combination. The treatments were undertaken with RPMI, progesterone, RU486 and progesterone + RU486. The increase in elafin expression in the presence of progesterone is significantly increased for treatment with IL-1β (red; $P<0.01$) and IL-1β + TNFα (blue; $P<0.001$). Treatment in the presence of progesterone + RU486 increases elafin expression significantly with IL-1β (red; $P<0.001$) and IL-1β + TNFα (blue; $P<0.001$). Data presented as relative to an untreated control (progesterone and RU486 are in an ethanol solution thus, controls were treated with an equal volume of ethanol), and represents 3 separate time points (4, 8 & 12 hr), n=5.

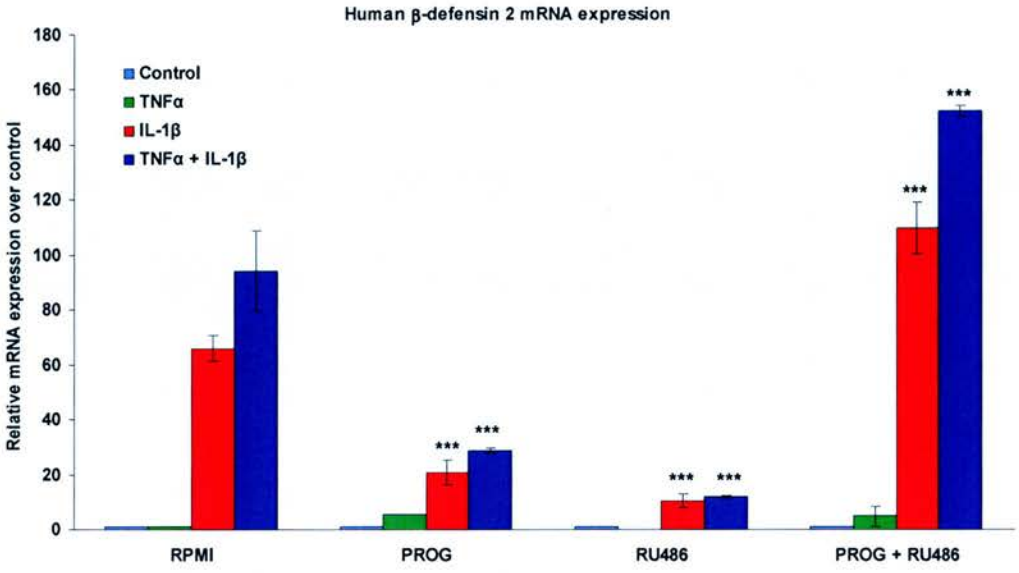


Figure 3.8.3.3 Human β-defensin 2 mRNA expression in Hec-1A cells treated with the pro-inflammatory cytokines TNFα, IL-1β and in combination. The treatments were undertaken with RPMI, progesterone, RU486 and progesterone + RU486. The decrease in hBD2 expression in the presence of progesterone is significant for treatment with IL-1β (red; $P<0.01$) and IL-1β + TNFα (blue; $P<0.001$). In the presence of RU486, there is significant decrease in hBD2 mRNA in response to IL-1β (red; $P<0.001$) and IL-1β + TNFα (blue; $P<0.001$). Treatment in the presence of progesterone + RU486 increases hBD2 expression significantly with IL-1β (red; $P<0.001$) and IL-1β + TNFα (blue; $P<0.001$). Data presented as relative to an untreated control (progesterone and RU486 are in an ethanol solution thus, controls were treated with an equal volume of ethanol), and represents 3 separate time points (4, 8 & 12 hr), n=5.

3.9 Discussion

The effect of the sex steroid progesterone and the antagonist RU486 upon the expression of natural antimicrobials in response to inflammatory mediators in the Hec-1A cells was examined and is summarised in table 3.9.1. A comparison with the published observations in primary endometrial material is summarised in table 3.9.2.

Hec-1A cells	**RPMI**	**Progesterone**	**RU486**	**Progesterone + RU486**
Elafin	↑	↑↑	No change	↑↑↑
Granulysin	-	↑↑	n/a	n/a
hBD2	↑	↓↓	↓↓↓	↑↑
SLPI	↑	↑↑	↓	↓

Figure 3.9.1 Elafin, hBD2 and SLPI mRNA was expressed in response to TNFα + IL-1β. Granulysin mRNA was undetected until the addition of progesterone, which also upregulated elafin and SLPI over the levels observed in RPMI. Human β-defensin 2 was downregulated in the presence of progesterone. Elafin mRNA levels were unchanged in the presence of RU486 when compared to RPMI, whilst hBD2 was greatly decreased and SLPI slightly decreased. Progesterone + RU486 greatly increased the level of elafin mRNA, increases hBD2 level above that observed for RPMI and decreases SLPI below the observed RPMI levels of SLPI.

Endometrium	Menstrual	Mid-secretory	Late - secretory	Ref.
Elafin	↑↑	-	↑	(King, Critchley et al. 2003)
Granulysin	-	-	↑↑	(King, Critchley et al. 2003)
hBD2	↑↑	-	-	(Fleming, King et al. 2003)
SLPI	-	↑↑	-	(King, Critchley et al. 2000)

Figure 3.9.2 Summary of natural antimicrobial expression in primary endometrial material across the menstrual cycle.

It has been previously shown that elafin is maximally expressed during the menstrual phase of the menstrual cycle, when the circulatory concentration of progesterone is low (King, Critchley et al. 2003). This may be suggestive of progesterone being inhibitory upon the expression of elafin. This was previously investigated further with the uterine epithelial cell lines ishikawa and HeLa, which have been shown not to express the nuclear progesterone receptor and were thus, not considered to be good models for progesterone responsiveness. The breast epithelial cell line T47D has been demonstrated to constitutively express the nuclear progesterone receptor (Horwitz, Mockus et al. 1982) and have been utilised in the study of elafin and SLPI (King, Morgan et al. 2003). King et al, demonstrated that there was no effect upon elafin expression with the addition of progesterone with the T47D cell line and suggested that the cyclic dependence of elafin expression in the endometrium may be as an indirect consequence of progesterone mediated regulation of other inflammatory mediators (King, Morgan et al. 2003). The endometrial epithelial cell line Hec-1A has been shown to express the nuclear progesterone receptor (Di Nezza,

Jobling et al. 2003). It has been demonstrated in this chapter that the Hec-1A cell line shows an increase in elafin mRNA expression in response to treatment with progesterone (figure 3.8.1.1 and 3.8.3.2). This is in contrast with the data presented in chapter 5 and reported previously (King, Critchley et al. 2003), where elafin is expressed in primary endometrial material obtained when circulating levels of progesterone were low. Primary endometrial material is made up of different cell types the major components being epithelial and stromal cells. Thus, it could be that the role of progesterone is indirect and acts via paracrine factor(s), which are not present when treating an epithelial cell line in culture. The role of stromal cells upon the epithelial expression of natural antimicrobials is addressed in chapter 4.

The treatment of Hec-1A cells with progesterone also demonstrated a significant increase in the expression of granulysin mainly a product of T lymphocytes and natural killer cells (Krensky 2000), and has not previously been shown to be expressed in an epithelial cell line. In primary endometrial material, granulysin was found to be maximally expressed during the late secretory phase of the menstrual cycle concurrent with the withdrawal of progesterone (King, Critchley et al. 2003) and the influx of NK cells (Fleming, King et al. 2003). The greatest level of granulysin mRNA in the Hec-1A cells was observed with the combined treatment of progesterone and IL-1β. The consistently observed synergism observed with the addition of other inflammatory mediators with IL-1β has not been repeated with respect to granulysin expression (figure 3.8.2.1). The data suggest that the addition of TNFα, LPS or LTA inhibits the level of granulysin expression observed with IL-1β alone. In order to further understand the effect of progesterone and inflammatory

mediators upon the epithelial expression of granulysin, further experiments with TNFα, LPS and LTA without IL-1β would be necessary.

SLPI is maximally expressed during the mid secretory phase of the menstrual cycle which is concurrent with a high circulating concentration of progesterone (King, Critchley et al. 2000). The increase in SLPI expression in response to progesterone was also shown with the treatment of cervical explants (Denison, Calder et al. 1999). The treatment of the cell line T47D with progesterone and inflammatory mediators gave rise to an increase in SLPI expression (King, Morgan et al. 2003). The data presented herein demonstrates that SLPI is expressed by the Hec-1A cell line and is also increased in response to TNFα + IL-1β in the presence of progesterone (figure 3.8.3.1). This increase was prevented with the addition of the progesterone antagonist RU486. SLPI has been described as a progesterone regulated gene and was shown to possess a progesterone response element (PRE) (King, Morgan et al. 2003). The Hec-1A cell line is derived from glandular epithelium and SLPI has been shown to be predominantly expressed in the glands of the endometrium (King, Critchley et al. 2000). Thus, the Hec-1A cells represent a fair model for the *in vitro* investigation in to the role of SLPI in the endometrium.

The treatment of Hec-1A cells with the progesterone antagonist RU486 increased the expression of elafin in response to IL-1β, 10-point over RPMI (figure 3.8.3.2). There was no change observed upon the IL-1β + TNFα combination with the addition of RU486, but, IL-1β alone produced a higher level of elafin (not significant). The presence of both progesterone and RU486 enhanced the response to IL-1β and IL-1β

+ TNFα over the increase observed in the presence of progesterone. This may suggest that the withdrawal of progesterone acts as a stimulator for the increase in elafin expression and would support the observation that elafin is increased in the late secretory – perimenstrual phase, when progesterone levels are in decline (King, Critchley et al. 2003). However, as was observed in the presence of RU486, elafin expression is maximal in response to treatment with IL-1β as opposed to IL-1β + TNFα in the presence of RU486 + progesterone. There is also a slight decrease in elafin in response to treatment with TNFα in the presence of RU486 and there could be an inhibitory effect on TNFα, thus explaining the loss of synergism between TNFα and IL-1β.

Human β-defensin 2 mRNA expression was inhibited when treated with TNFα and IL-1β in the presence of progesterone (figure 3.8.3.3) in the Hec-1A cells. Human β-defensin 2 has been shown to be maximally expressed during the menstrual phase (Fleming, King et al. 2003), which supports the data observed with the Hec-1A cells. This may be suggestive of a direct inhibitory role for progesterone in the expression of hBD2 or an indirect effect via other progesterone mediated factor(s). As was observed for elafin, there is a further increase in hBD2 mRNA expression in the presence of RU486 and progesterone, which may also indicate a response to progesterone withdrawal.

In summary, the presence of nuclear progesterone receptors in the Hec-1A endometrial epithelial cell line, allows for the analysis of effects upon antimicrobials mediated by progesterone. Herein it has been shown that progesterone upregulates SLPI, elafin and granulysin mRNA, whilst downregulating hBD2. The upregulation of SLPI is in support of observations from primary endometrial samples (table 3.9.2), where SLPI is maximal during the mid secretory phase (high progesterone). Similarly, the progesterone mediated downregulation of hBD2 may be predicted as this antimicrobial has been shown to be maximally expressed during menstrual phase (low progesterone) endometrial biopsies (table 3.9.2). However, there was an upregulation of elafin in the presence of progesterone and this was unexpected as elafin has also been demonstrated to be increased (late secretory) and maximally expressed during the menstrual phase in endometrial biopsies. It may be that progesterone exerts an effect via other mediators or paracrine factor(s), and this is subject to further investigation in chapter 4. The treatment of Hec-1A cells with TNFα + IL-1β in the presence of progesterone and the progesterone antagonist RU486 increased the expression of elafin and hBD2 when compared to the levels observed with progesterone alone. This may simulate progesterone withdrawal and in turn brings about the upregulation of other factors that may give rise to the increase in antimicrobial expression.

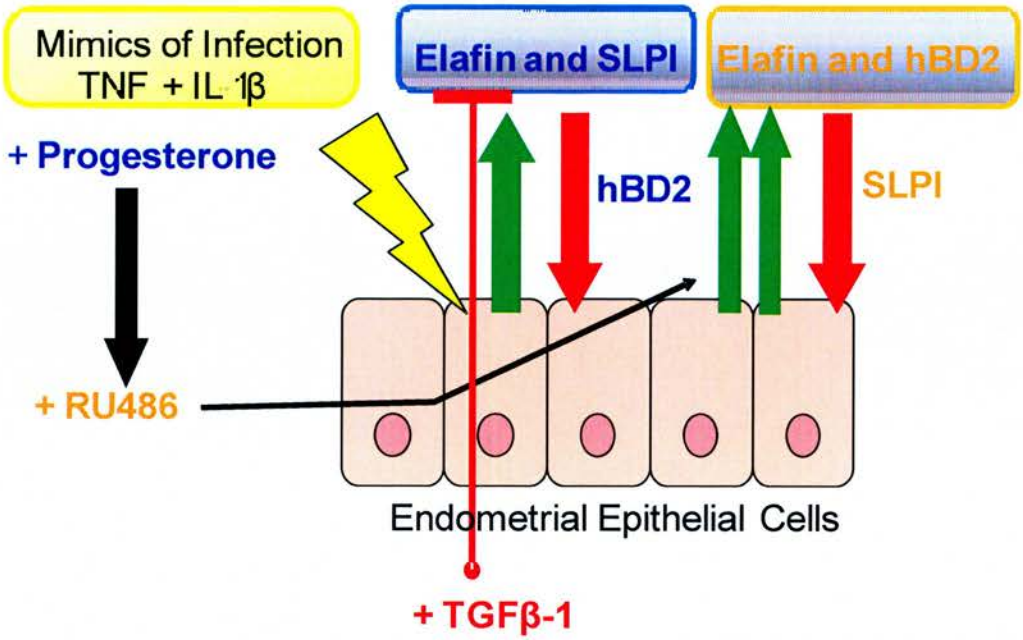


Figure 3.9.2 The addition of progesterone (blue) caused a further increase in expression of elafin and SLPI. HBD2 was downregulated with the addition of progesterone. Mifepristone (RU486) treatment (orange) resulted in a further increase in elafin mRNA expression and restored hBD2 expression. SLPI was downregulated in response to mifepristone.

Chapter 4:

Paracrine influences of endometrial epithelial stromal cell interaction in the expression of elafin

Chapter 4: Paracrine influences of endometrial epithelial-stromal cell interaction in the expression of elafin.

4.1 Introduction

Elafin is a serine protease inhibitor and is expressed by a number of epithelial and immune cells in response to injury or infection (Sallenave 2000; Hiemstra 2002; Sallenave 2002). Elafin has also been demonstrated to have antimicrobial actions against bacteria, fungi and viruses (Hiemstra, Maassen et al. 1996; Simpson, Maxwell et al. 1999; Sallenave 2002). The epithelial expression of elafin has been described in the endometrium (King, Critchley et al. 2003) and was confirmed in chapter 5 of the current thesis to be maximally expressed during the menstrual phase. It has also been observed that there is elafin expression within the neutrophil population of the endometrium during the menstrual phase (King, Critchley et al. 2003). Elafin expression was also observed within the leukocyte population of the Fallopian tube (chapter 6) and in the decidua from women with an ectopic gestation (chapter 7).

The use of cell lines and *in vitro* models have demonstrated the inducibility of elafin in the presence of the inflammatory cytokines TNFα and IL-1 in the lung (Sallenave, Shulmann et al. 1994), keratinocytes (Tanaka, Fujioka et al. 2000) endometrium (King, Fleming et al. 2002) and mammary cells (Zhang, Magit et al. 1997). In chapter 3 of the current thesis it was shown that elafin expression is inducible in the glandular endometrial epithelial cell line, Hec-1A.

The human endometrium is composed of a number of different cell types and subject to change in response to different signalling and interaction mechanisms. The interaction between these different cell types is becoming increasingly recognised as important to the function of the endometrium. The interaction between the stroma and the extracellular matrix (ECM) is important for the regulation of cell proliferation and growth (Cunha, Bigsby et al. 1985). This interaction is also required for the process of embryonic cell differentiation (Arnold, Lessey et al. 2002). The interaction between endometrial stromal and epithelial cells has also been identified as important for progesterone mediated events (Kurita, Wang et al. 2001; Cunha, Cooke et al. 2004).

In chapter 3, it was shown that the inflammatory stimulus of Hec-1A cells in the presence of progesterone increased the expression of elafin (chapter 3; figure 3.8.1.1). This was in contrast to previous observations that elafin is maximally expressed during the menstrual phase, when circulating progesterone levels are low (King, Critchley et al. 2003) and demonstrated herein (chapter 5; figure 5.3.2.1). This led to the assumption that progesterone was inhibitory either directly or indirectly for the expression of elafin. However, *in vitro* studies using the T47D cell line showed elafin mRNA to be unaffected by the presence of progesterone. The endometrial biopsies investigated for elafin expression consisted of both stromal and epithelial components. This may be suggestive of a role for paracrine factor(s) of stromal origin which may influence the epithelial expression of elafin in response to progesterone. The influence of stromal mediated paracrine factors upon the

expression of natural antimicrobials has not previously been described and will be the focus of the current chapter.

In order to investigate the role of stromal mediated factors upon the epithelial expression of elafin, an *in vitro* model was deemed necessary. In the current study, two approaches where adopted. The **first** utilised conditioned media which was removed from stromal cells and placed upon epithelial cells prior to treatment with inflammatory stimuli described in section 4.2.1.1 and summarised in figure 4.2.1.1. The **second** method employed involved the use of a co-culture set-up as described in chapter 2 (section 2.2.5) and in section 4.2.1.2 and figure 4.2.1.2 of the current chapter. The latter method allowed for 'two-way' communication between the endometrial stromal cells and the epithelial Hec-1A cell line.

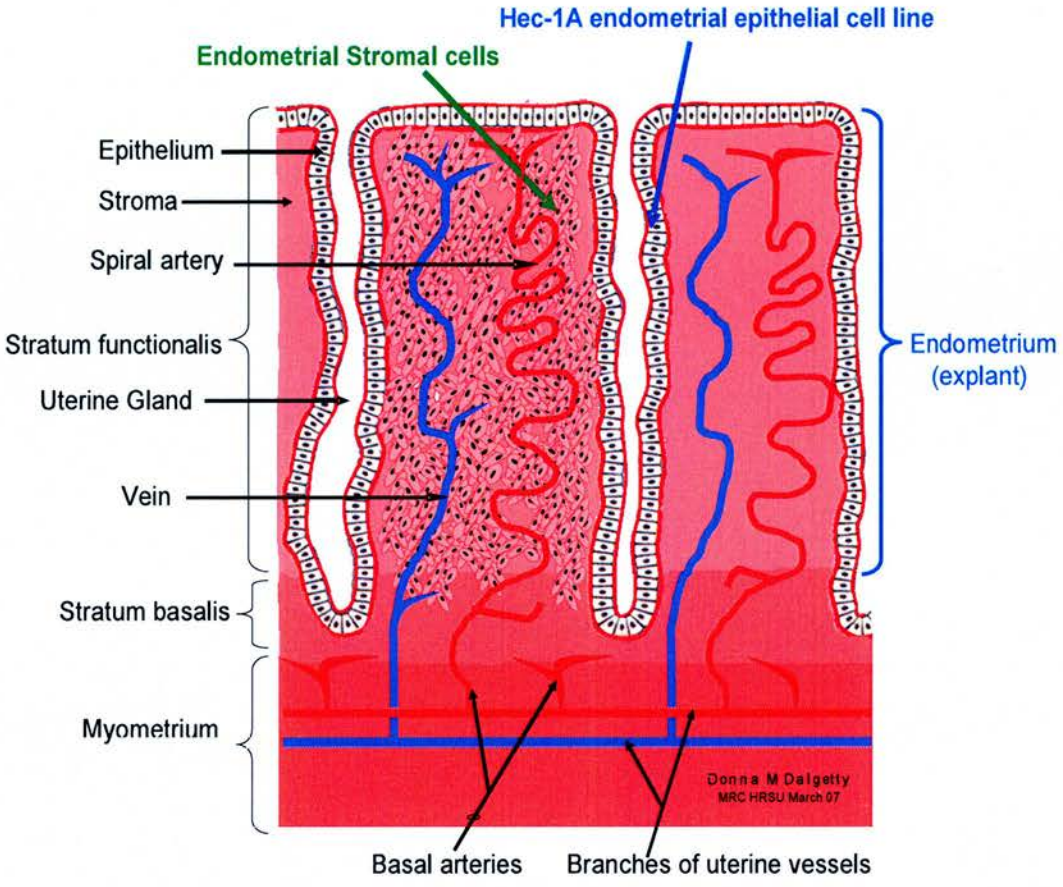


Figure 4.1.1 A schematic diagram showing the structure and positional arrangement of the stromal and epithelial cells of the endometrium. The Hec-1A cell line was derived from the glandular epithelium, whilst the stromal cells were obtained from primary culture as described in section 2.1.1. The endometrial explants examined for natural antimicrobial expression (chapter 5), are also demonstrated and show the presence of both stroma and epithelia.

Aims:

- Investigate whether there are paracrine factors in the epithelial expression of elafin [as described in chapt 3]
- Investigate paracrine mediated effects in the presence of progesterone
- Suggest candidate paracrine factors involved in any elafin relevant interaction between epithelial and stromal cells.

4.2 Materials and Methods

4.2.1 Cell culture

The human endometrial epithelial cell line Hec-1A (human endometrial cancer-one) (Kuramoto, Tamura et al. 1972), were cultured as described in 2.2.3. The stromal cells were derived from primary endometrial biopsies and were prepared as described in section 2.2.1. The treatments used for this chapter are detailed in the table below (Table 4.2.1.1).

Treatment	Concentration used
Control	n/a
IL-1β	5 ng/ml
TNFα	5 μg/ml
Progesterone	10^{-6} M

Table 4.2.1.1 Details of treatment of Hec-1A endometrial cell line. Supplier information is contained within appendix I.

4.2.1.1 Conditioned culture media

Conditioned culture media was prepared as follows: complete RPMI culture media with or without progesterone was placed upon endometrial stromal cells (70% confluent) and incubated for 24 hr. The media was then removed and filter sterilised via a vacuum pump (Nalgene) and supplemented with new media with a ratio 50:50.

The filtered media was replenished to ensure that the cells were sufficiently nourished. The media was added to the Hec-1A cells supplemented with the relevant treatment as detailed for each relevant experiment (TNFα, IL-1β and TNFα + IL-β). The process of media preparation is summarised in figure 4.2.1.1.

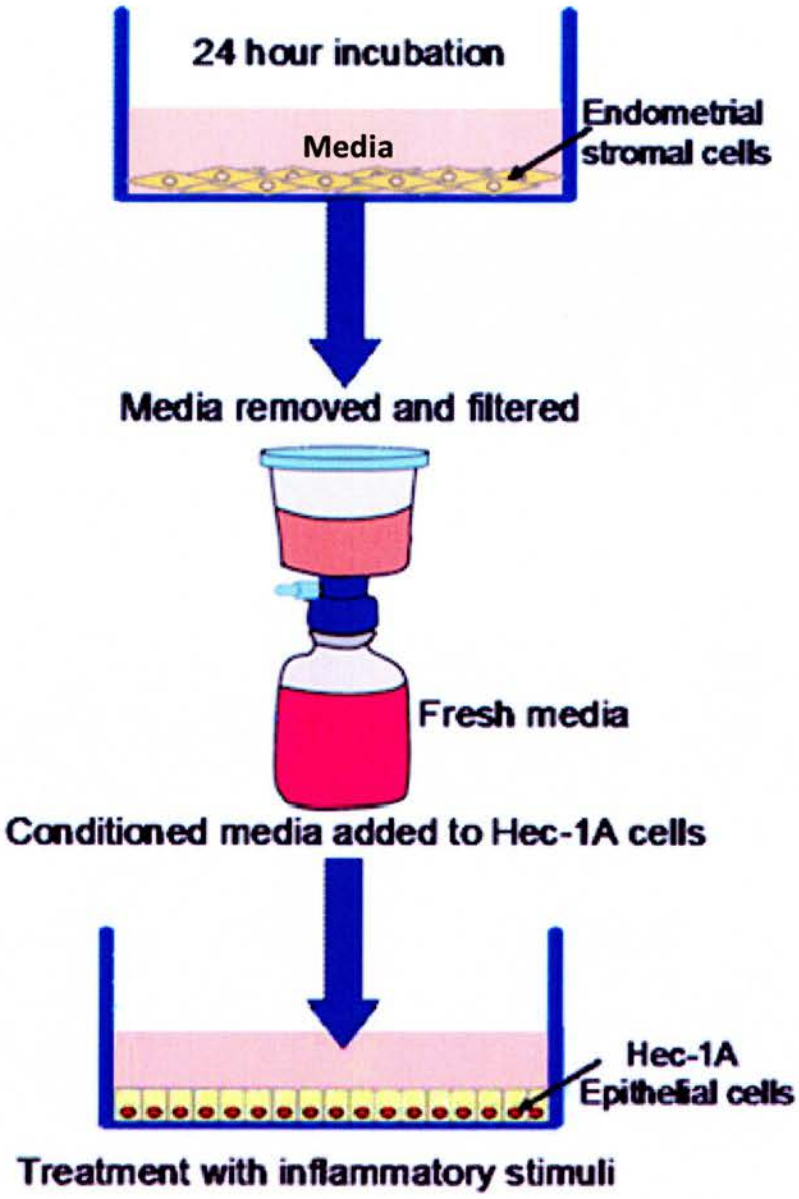


Figure 4.2.1.1 Schematic summary of the preparation of stromal conditioned media. Culture media (RPMI) with or without progesterone was incubated for 24 hr with endometrial stromal cells at 70% confluence. The media was then poured off, filtered and replenished with new culture media (50:50). The media was then utilised for the culture and treatment of Hec-1A epithelial cells.

4.2.1.2 Co-culture of endometrial stromal cells and the Hec-1A epithelial cell line

Co-culture experiments were made up of the following: endometrial stromal cells (ESCs) were seeded into 6 well culture plates and allowed to reach confluence. After attachment the media containing serum and hormones was removed and replaced with serum free RPMI. Hec-1A epithelial cell line was seeded ($1.3x10^5$ cells/ml) into the interior of transwell culture inserts in 2% cRPMI and allowed to adhere. The inserts containing the epithelial cells were then placed in the wells containing the stromal cells and the media replaced prior to treatment with inflammatory mimics (IL-1β and TNFα) with or without steroid hormones (progesterone). Co-culture controls without treatment and single culture controls for both ESC and Hec-1A (treated and untreated) cells were included throughout. The co-culture set-up and methods of treatment are demonstrated in chapter 2; figure 2.2.5.1, and indicated where relevant in the results section by numbers which are presented as a key to the figure below (figure 4.2.1.2).

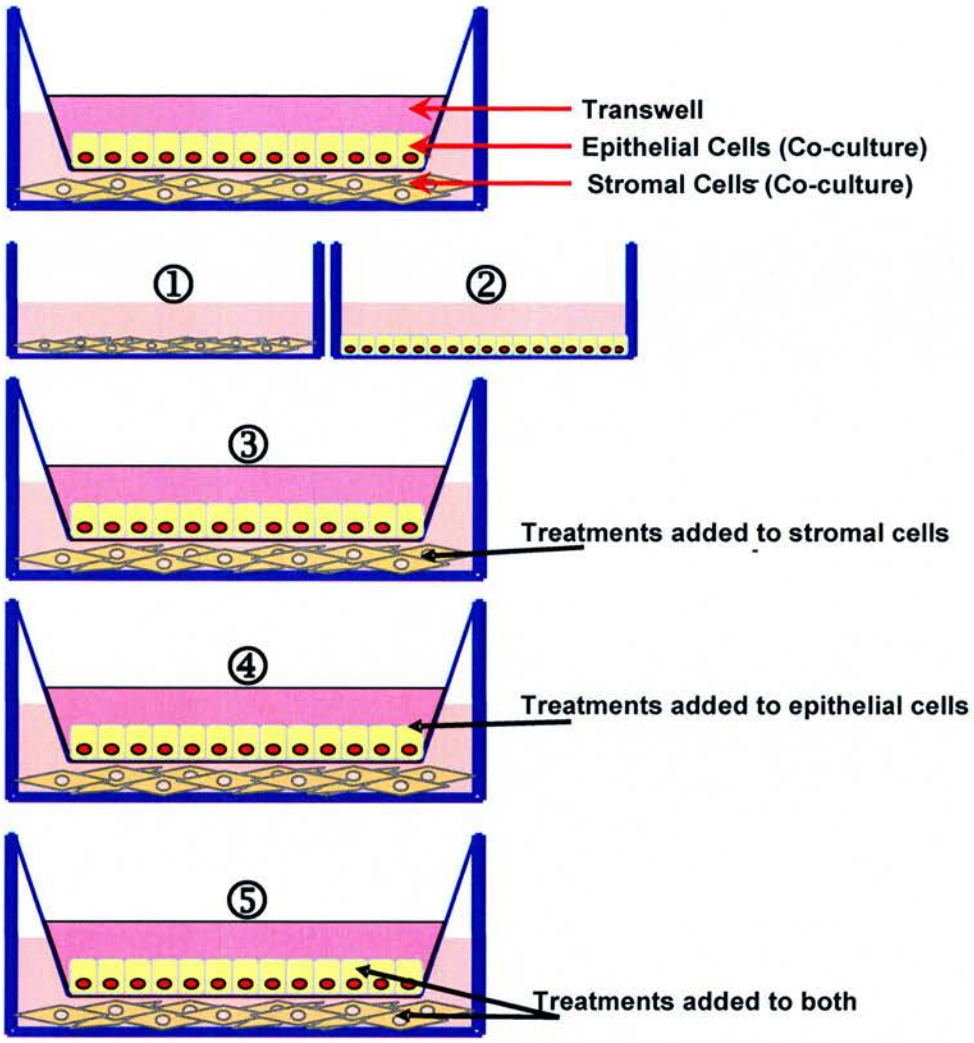


Figure 4.2.1.2 Diagram depicting the different treatment methods used with co-culture plates. The circled numbers are included within the results to indicate which of the above arrangements was employed. ① Stromal cells alone; ② epithelial cells alone; ③ co-culture plate where the stromal compartment is treated; ④ co-culture plate where the epithelial compartment is treated; ⑤ co-culture plate where the media is treated prior to being placed in both compartments equally.

4.2.2 RNA extraction and Q-RT-PCR

Following treatment and incubation for the relevant time point, the RNA was extracted from the cells and cDNA prepared as described in section 2.3. Elafin mRNA levels were measured in these cDNA samples by quantitative PCR (2.3). The sequence details of the primer-probe sets used are detailed in table 2.3.3.1, materials and methods.

4.2.3 ELISA

The culture supernatants were removed from the cells, sterile filtered and the amount of elafin protein was determined via an ELISA as detailed in section 2.4.

4.2.4 Statistical analysis

The PCR results in this chapter were analysed by ANOVA for significant difference. Fisher's protected least significant difference (PLSD) was used to assign individual differences (PRISM).

Each n=1 is equal to the mean of 3 individual time points (4, 8 and 12 hr), in order to account for the variances in expression across time and allow for comparison on the basis of treatment conditions alone.

4.3 Results

4.3.1 The effect of stromal conditioned media upon the expression of elafin mRNA in Hec-1A cells treated with TNFα and IL-1β.

In order to investigate the role of stromal mediated paracrine factors upon the expression of elafin, culture media previously cultured with endometrial stromal cells was placed upon Hec-1A cells concurrently with treatment with pro-inflammatory cytokines, TNFα and IL-1β. This was undertaken both with and without the presence of progesterone as described in section 4.2.1.

Elafin mRNA, figure 4.3.1.1, was increased when treated with TNFα, IL-β and TNFα + IL-1β, in the presence of stromal conditioned media. Elafin mRNA was 11.3 point greater with TNFα, 301-point greater with IL-1β ($P<0.001$), and 26-point greater with TNFα + IL-1β, in the presence of stromal conditioned media, when compared to RPMI alone (n=6; derived as explained in section 4.2.4). The synergistic response of elafin mRNA expression in response to treatment with TNFα + IL-1β in less than that observed for the treatment with IL-1β alone in the presence of stromal conditioned media. Elafin mRNA is 237-point greater in response to IL-1β, when compared with the addition of TNFα, in the presence of stromal conditioned media.

In the presence of progesterone (10^{-6} M), stromal conditioned media reduced the expression of elafin mRNA (figure 4.3.1.2), in response to treatment with TNFα, TNFα + IL-1β ($P<0.001$). Elafin mRNA was decreased 13-point in response to

TNFα and 128-point in response to treatment with TNFα + IL-1β ($P<0.001$), in the presence of stromal conditioned media with progesterone. However, the addition of IL-1β in the stromal conditioned media increased elafin mRNA expression (27-point) when compared to unconditioned progesterone treated media. Furthermore as with figure 4.3.1.1, the synergistic action of TNFα + IL-1β upon elafin mRNA, was reduced below that observed for IL1β (22-point) in the presence of stromal conditioned media with progesterone.

Relative increase over untreated controls	RPMI culture media	Stromal conditioned RPMI	Progesterone (10^{-8} M) RPMI	Stromal conditioned prog RPMI
TNFα	6	17	21	8
IL-1β	70	371	75	102
TNFα + IL-1β	108	134	208	79

Table 4.3.1.1 Summary of elafin relative mRNA results shown in figures 4.3.1.1 and 4.3.1.2

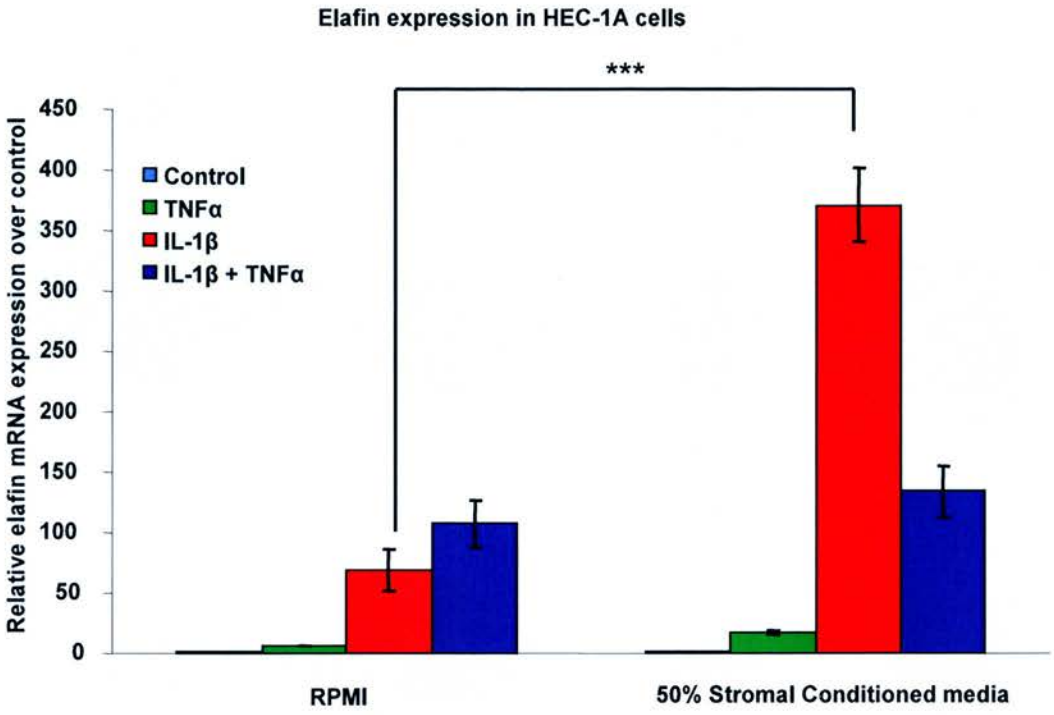


Figure 4.3.1.1 Elafin mRNA expression in Hec-1A cells in response to treatment with inflammatory cytokines, TNFα (green), IL-1β (red) and TNFα + IL1β (blue), with and without the presence of stromal conditioned media. Media that was removed after 24 hour incubation with endometrial stromal cells was supplemented with 50% RPMI (detailed in section 4.2.1). Elafin mRNA was significantly increased in response to treatment with IL-1β in the presence of stromal conditioned media compared to RPMI alone ($P<0.001$). Data are presented as relevant to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ±s.e.m. Each experiment (n=6) was performed at 4, 8 and 12 hr; the 3 time points were averaged, taken as n=1, and served to allow for variances in response to time.

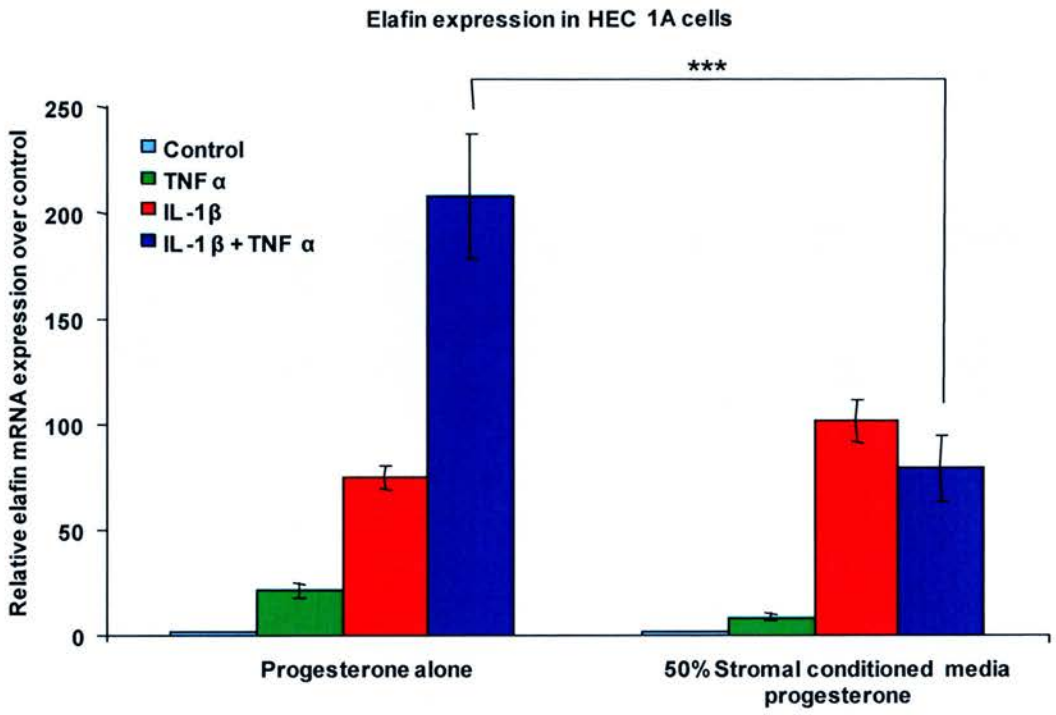


Figure 4.3.1.2 Elafin mRNA expression in Hec-1A cells in response to treatment in progesterone (10^{-6} M) and TNFα (green), IL-1β (red) and TNFα + IL1β (blue), with and without the presence of stromal conditioned media. Progesterone supplemented media, which was removed after 24 hour incubation with endometrial stromal cells was supplemented with 50% RPMI (detailed in section 4.2.1). Elafin mRNA was significantly decreased when treated with TNFα + IL-1β in the presence of stromal conditioned media when compared to RPMI alone ($P<0.001$). Data are presented as relevant to a control (untreated Hec-1A cells for each time point); given a nominal value of 1, mean ±s.e.m. Each experiment (n=6) was performed at 4, 8 and 12 hr; the 3 time points were averaged, taken as n=1 and served to allow for variances in response to time.

4.3.2 The effect of the co-culture of endometrial stromal cells with the Hec-1A epithelial cell line when treated with pro-inflammatory cytokines upon the expression of elafin mRNA.

Further to the data obtained in section 4.3.1 which indicated the presence of stromal mediated factors, it was deemed necessary to examine the effect of two-way communication between endometrial stromal cells and Hec1A epithelial cells, thus, a co-culture set-up was investigated (figure 4.2.1.2).

Hec-1A expression of elafin mRNA, figure 4.3.2.1 (n=2; derived as described in section 4.2.4), was increased when treated with TNFα, IL-1β and TNFα + IL-1β, in the presence of stromal cells in co-culture, when compared to Hec1A cells alone. Elafin mRNA was increased 3-point with TNFα, 19-point with IL1β, and 20-point with TNFα + IL-1β in the presence of stromal cells. There was no notable difference in the level of elafin mRNA expression in stromal cells in the presence of epithelial cells for any of the pro-inflammatory cytokines.

In the presence of progesterone the co-culture of Hec-1A cells with endometrial stromal cells greatly decreases the level of elafin mRNA expression in response to treatment with TNFα (1.5-point), IL1β (18-point) and TNFα + IL-1β (83-point) in comparison with epithelial cells alone, figure 4.3.2.2 (n=2). The stromal cells did not demonstrate any difference in the expression of elafin mRNA in co-culture or alone. The stromal cells only express elafin mRNA at a very low level.

Further analysis of the co-culture model was undertaken to examine the effect of treating the individual components of the set-up with inflammatory stimuli. Therefore, the effect of treating only the stromal cells or epithelial cells in co-culture was examined for changes in elafin mRNA.

Elafin mRNA was increased in Hec-1A cells in response to the treatment of stromal cells in co-culture with TNFα (1.5-point), IL-1β (16-point) and TNFα + IL-1β (23-point), when compared with the same treatment of epithelial cells alone figure 4.3.2.3. The treatment of the stromal cells also increased the stromal expression of elafin mRNA, TNFα, 3.5-point; IL-1β, 6-point and TNFα + IL-1β, 9-point, (n=2).

Treatment of the Hec-1A cells with inflammatory stimuli increased the expression of elafin mRNA from both the Hec-1A cells and the stromal cells when compared with the level of expression observed for each of the cell types cultured alone, figure 4.3.2.3, (n=2). The stromal cells increased elafin mRNA expression in response to TNFα (3-point), IL-1β (6-point) and TNFα + IL-1β (10-point), when Hec-1A cells were treated in co-culture in comparison with stromal cells alone. Elafin mRNA expression was also increased in the Hec-1A cells treated in co-culture with stromal cells, IL-1β (9-point) and TNFα + IL-1β (11-point). The treatment of stromal cells in co-culture demonstrated the greatest increase in elafin mRNA expression in the Hec-1A cells.

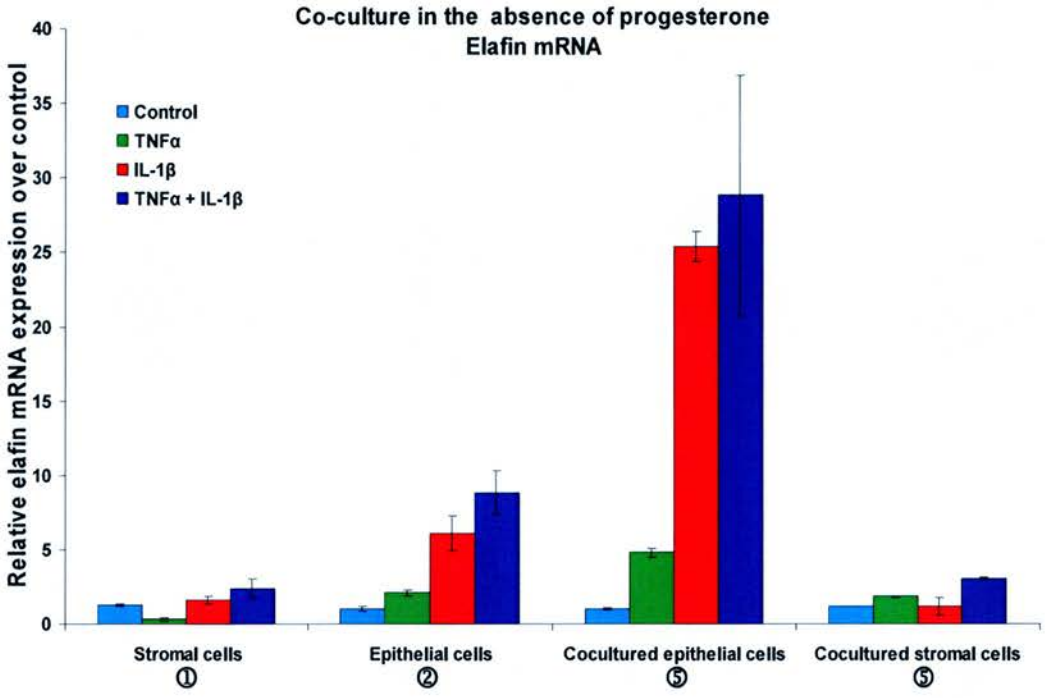


Figure 4.3.2.1 Elafin mRNA expression in Hec-1A and stromal cells in response to treatment with pro-inflammatory cytokines, TNFα (green), IL-1β (red) and TNFα + IL-1β (blue). The culture plate was set-up as shown in figure 4.2.1.2, with the numbers indicating the precise arrangement. (1) Endometrial stromal cells (ESC) alone; (2) Hec-1A epithelial cell line and (5) co-culture containing ESCs and Hec1A cells, the media was treated prior to the addition to both compartments concurrently. ESC cells showed no difference in elafin mRNA expression when treated in co-culture to cells alone. Hec-1A cells demonstrated an increase in elafin mRNA in response to inflammatory cytokines in the co-culture set-up. Data are presented as relevant to a control (untreated ESC or Hec-1A); given a nominal value of 1, mean ± s.e.m. Each experiment (n=2) was performed at 4, 8 and 12 hr; the 3 time points were averaged, taken as n=1 and served to allow for variances in response to time.

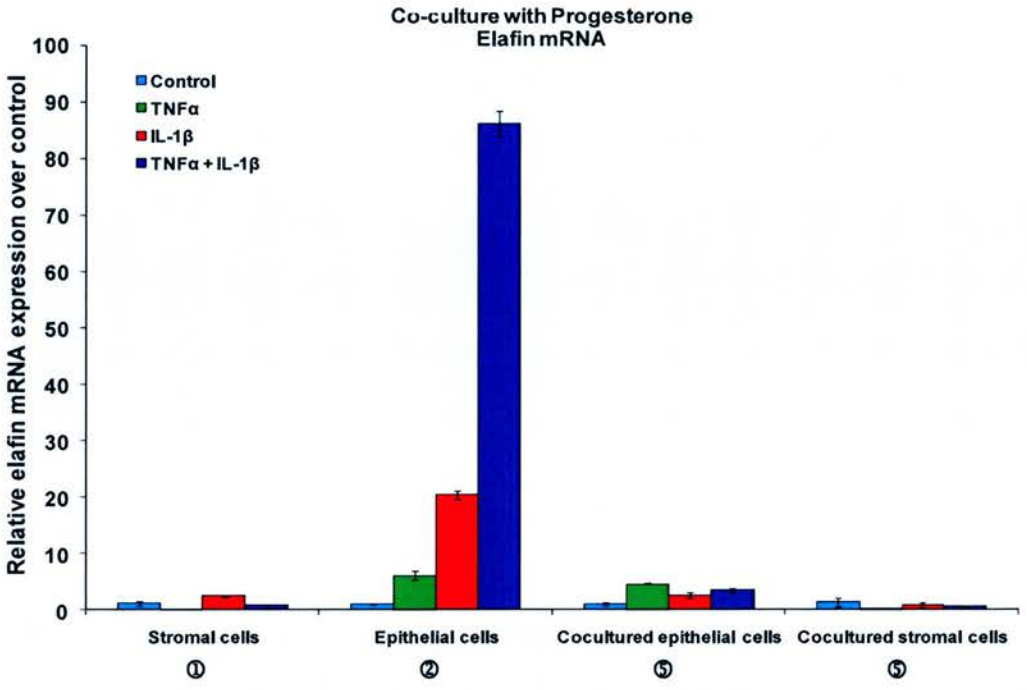


Figure 4.3.2.2 Elafin mRNA expression in Hec-1A and stromal cells in response to treatment with pro-inflammatory cytokines, TNFα (green), IL-1β (red) and TNFα + IL-1β (blue), in the presence of progesterone (10^{-6} M). The culture plate was set-up as shown in figure 4.2.1.2, with the numbers indicating the precise arrangement. (1) Endometrial stromal cells (ESC) alone; (2) Hec-1A epithelial cell line and (5) co-culture containing ESCs and Hec1A cells, the media was treated prior to the addition to both compartments concurrently. ESC cells showed no difference in elafin mRNA expression when treated in co-culture to cells alone. Hec-1A cells demonstrated a decrease in elafin mRNA in response to progesterone and inflammatory cytokines in the co-culture set-up. Data are presented as relevant to a control (untreated ESC or Hec-1A); given a nominal value of 1, mean ± s.e.m. Each experiment (n=2) was performed at 4, 8 and 12 hr; the 3 time points were averaged, taken as n=1 and served to allow for variances in response to time.

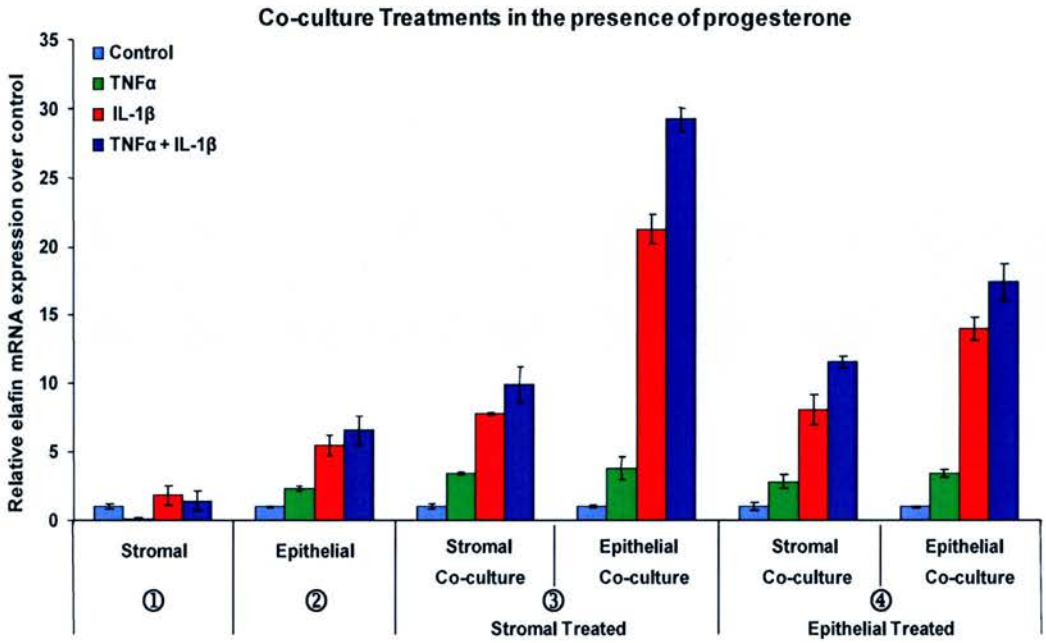


Figure 4.3.2.3 Elafin mRNA expression in Hec-1A and stromal cells in response to treatment with pro-inflammatory cytokines, TNFα (green), IL-1β (red) and TNFα + IL-1β (blue), in the presence of progesterone (10^{-6} M). The culture plate was set-up as shown in figure 4.2.1.1, with the numbers indicating the precise arrangement. (1) Endometrial stromal cells (ESC) alone; (2) Hec-1A epithelial cell line and (4) co-culture containing ESCs and Hec1A cells, the media was treated prior to the addition to both compartments concurrently. ESC cells showed no difference in elafin mRNA expression when treated in co-culture to cells alone. Hec-1A cells demonstrated a increase in elafin mRNA in response to progesterone and inflammatory cytokines in the co-culture set-up. Data are presented as relevant to a control (untreated ESC or Hec-1A); given a nominal value of 1, mean ± s.e.m. Each experiment (n=2) was performed at 4, 8 and 12 hr; the 3 time points were averaged, taken as n=1 and served to allow for variances in response to time.

4.3.3 The effects of the co-culture of endometrial stromal cells with the Hec-1A epithelial cell line and treatment with TNFα and IL-1β on the expression of elafin protein.

The expression of elafin protein (figure 4.3.3.1 – 4.3.3.3) in the presence of TNFα and IL-1β, with progesterone (blue) and without (purple) in the co-culture set-up described in figure 4.2.1.2, was found to be differential depending on where the culture media was sampled.

Treatment of the stromal cells (figure 4.3.3.1; n=2) with TNFα and IL-1β singly and in combination in RPMI demonstrated similar levels of elafin protein in the co-culture (stromal) and transwell (Hec-1A) culture media. In the presence of progesterone there was more elafin protein in the co-culture and transwell media, 170-point and 130-point, respectively, when treated with TNFα + IL-1β.

Treatment of the epithelial cells (figure 4.3.3.2; n=2) with TNFα and IL-1β was shown to increase the levels of elafin protein and lower levels were obtained in the presence of progesterone except in the transwell media treated with TNFα + IL-1β, where 284 pg/ml of elafin was detected with progesterone compared to 155 pg/ml. In the corresponding co-culture well the treatment of the Hec-1A cells with TNFα + IL-1β gave rise to 550 pg/ ml of elafin, and in the presence of progesterone this was reduced (200 pg/ml).

Treatment of the media prior to the addition to both cell compartments, figure 4.3.3.3 (n=2) gave a different pattern of elafin protein expression. The stromal co-culture

media in the presence of progesterone and treated with IL-1β contained more elafin protein (250 pg/ml) than without the presence of progesterone (105 pg/ml). Treatment with the combined cytokines in the presence of progesterone reduced the level of elafin protein (350 pg/ml) when compared to the level of elafin detected in the absence of progesterone (568 pg/ml). The media collected from the transwell (Hec-1A) contained less elafin protein than was detected in the stromal area. Treatment with IL-1β produced 88 pg/ml and this was found to be reduced in the presence of progesterone, 14 pg/ml. With the treatment with TNFα + IL-1β, 122 pg/ml of elafin protein was detected and this was found to be increased in the presence of progesterone, 252 pg/ml.

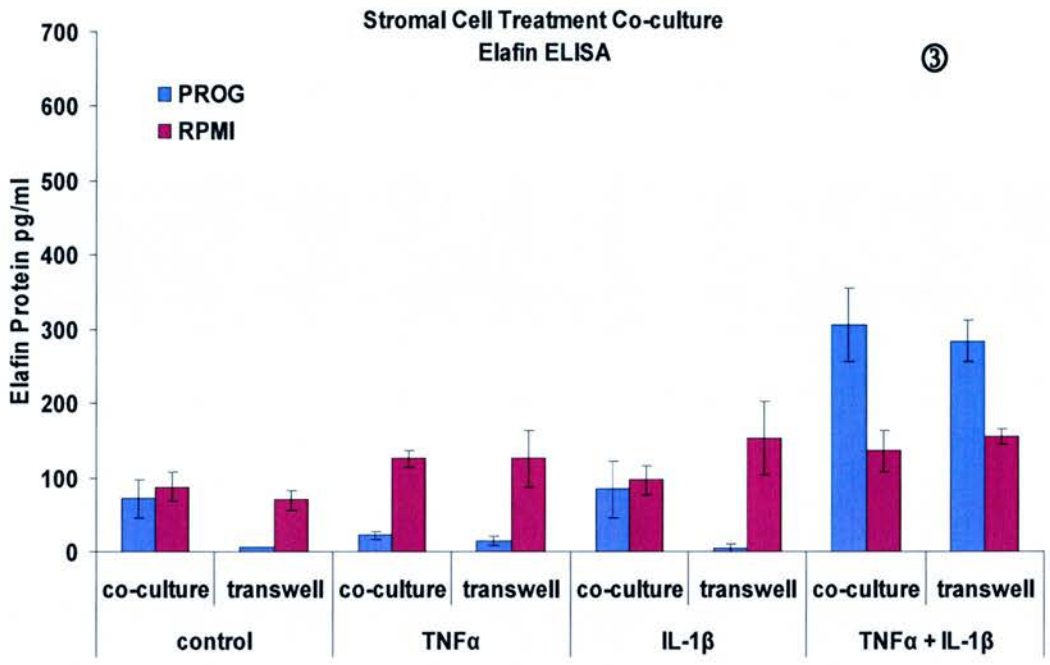


Figure 4.3.3.1 Elafin protein (pg/ml) detected within the culture supernatants from stromal and epithelial cells. The supernatants were collected from the stromal component (co-culture) and the Hec-1A cells (transwell). The stromal cells were treated with pro-inflammatory cytokines, TNFα, IL-1β and TNFα + IL-1β (figure 4.2.1.2 ③). The cells were treated with (blue) and without (purple), progesterone (10^{-6} M). An equal amount of ethanol was added to the cells without progesterone (the progesterone is made up with ethanol). NB. co-culture wells contain endometrial stromal cells and the transwells contained Hec-1A epithelial cells.

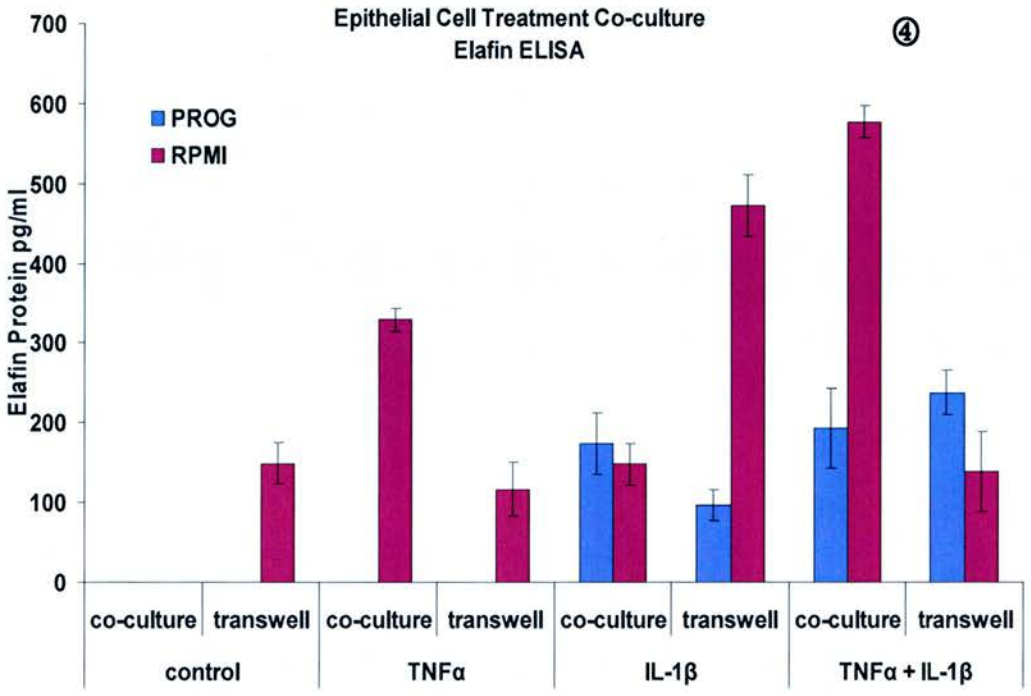


Figure 4.3.3.2 Elafin protein (pg/ml) detected within the culture supernatants from stromal and epithelial cells. The supernatants were collected from the stromal component (co-culture) and the Hec-1A cells (transwell). The Hec-1A epithelial cells were treated with pro-inflammatory cytokines, TNFα, IL-1β and TNFα + IL-1β (figure 4.2.1.2 ④). The cells were treated with (blue) and without (purple), progesterone (10^{-6} M). An equal amount of ethanol was added to the cells without progesterone as a control (the progesterone is made up with ethanol). NB. co-culture wells contain endometrial stromal cells and the transwells contained Hec-1A epithelial cells.

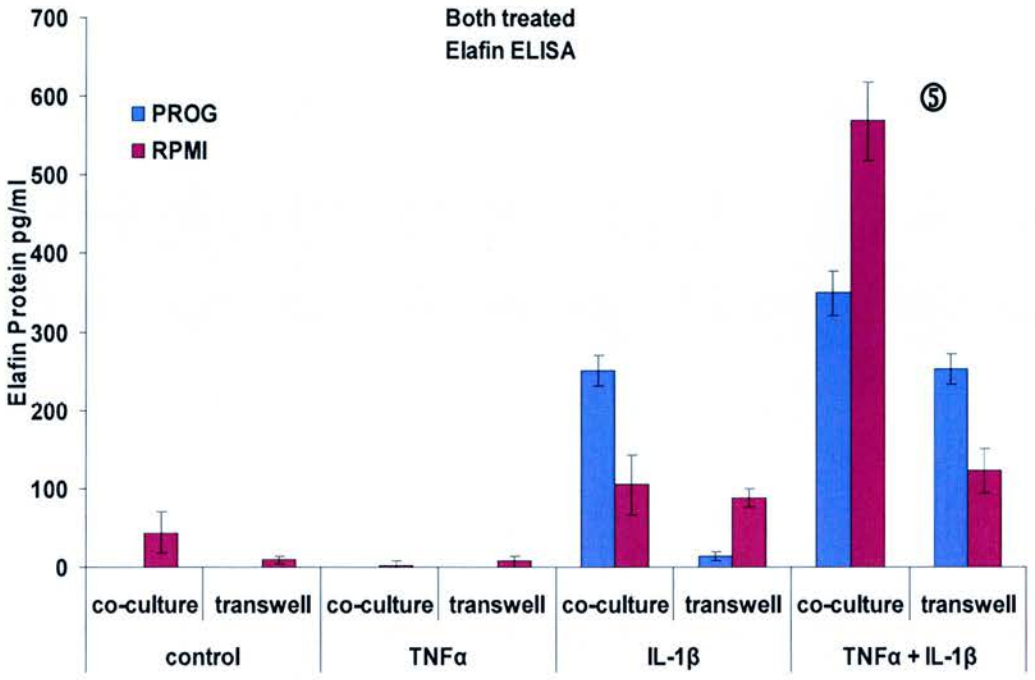


Figure 4.3.3.3 Elafin protein (pg/ml) detected within the culture supernatants from stromal and epithelial cells. The supernatants were collected from the stromal component (co-culture) and the Hec-1A cells (transwell). The media was treated with pro-inflammatory cytokines, TNFα, IL-1β and TNFα + IL-1β (figure 4.2.1.2 ⑤), prior to the addition to the culture plates. The treatments were undertaken with (blue) and without (purple), progesterone (10^{-6} M). An equal amount of ethanol was added to the cells without progesterone as a control (the progesterone is made up with ethanol). NB. co-culture wells contain endometrial stromal cells and the transwells contained Hec-1A epithelial cells.

Summary of results

1. Stromal derived media increases epithelial (Hec-1A) expression of elafin mRNA
2. Hec-1A expression of elafin mRNA is inhibited with the addition of progesterone + stromal derived media.
3. Co-culture of ESCs (endometrial stromal cells) and Hec-1A epithelial cells with and without progesterone produced similar data as stromal conditioned media experiments (1 & 2 above).
4. ESCs express elafin in the presence of Hec-1A cells, when treated directly (stromal compartment) or indirectly (transwell) with inflammatory stimuli
5. ESC treatment of co-cultures caused the greatest increase in elafin mRNA by Hec-1A cells.
6. Elafin protein was detected to be variable between compartments, suggesting that little culture media is exchanged.

4.4 Discussion

The investigation into the expression of elafin in the endometrium has been undertaken both within the current thesis and in previous studies, involving the use of primary endometrial explants collected from women across the menstrual cycle (King, Critchley et al. 2003) and in chapter 5 of the present thesis. The data obtained from these studies demonstrated that elafin was maximally expressed during the menstrual phase of the cycle, where circulating levels of progesterone are low (figure 5.3.2.1). This was suggestive that progesterone may directly or indirectly have an inhibitory role in the expression of elafin (King, Critchley et al. 2003). This was further predicted with the demonstration that SLPI contains a progesterone response element (PRE) and thus, is up-regulated directly in response to progesterone, and is maximally expressed during the mid secretory phase (high circulating levels of progesterone) (King, Critchley et al. 2003; King, Morgan et al. 2003). However, although in vitro cell line work confirmed the up-regulation of SLPI by progesterone, there was no effect described for elafin in the breast cancer cell line T47D (King, Morgan et al. 2003). The use of the Hec-1A endometrial epithelial cell line in chapter 3 of the current thesis, however, did give rise to a response in the presence of progesterone (figure 3.8.1.1), with an increase in elafin expression. The data obtained for the expression of elafin across the menstrual cycle, was from the analysis of endometrial explants (figure 4.1.1), which consists of a number of different cells, predominantly that of the stroma and epithelia. Thus, the effect of paracrine interaction between these two cell types upon the expression of elafin was investigated.

Initial investigations utilised cell conditioned media as shown in figure 4.2.1.1 (section 4.2.1.1). Briefly, the media was pre-conditioned with endometrial stromal cells for 24 hour, prior to being filtered and replenished with fresh media at a ratio of 50:50. This media was then placed upon the Hec-1A cell line, along with the pro-inflammatory cytokines TNFα and IL-1β. The addition of this stromal conditioned media was shown to increase the expression of elafin mRNA by the Hec-1A cell line. The expression of elafin mRNA in response to treatment with TNFα in stromal conditioned media was over 11-point increased and 26-point greater when treated with TNFα + IL-1β, when compared to unconditioned media (figure 4.3.1.1). However, it was the treatment of Hec-1A cells with IL-1β in the presence of stromal conditioned media which was demonstrated to give the greatest increase in elafin mRNA expression, 301-point greater ($P<0.001$). This is in contrast to the previously observed (chapter 3), synergistic response to treatment with both TNFα and IL-1β and as shown in the unconditioned media treatment for the same experiment (figure 4.3.1.1). This greater expression in elafin mRNA in response to IL-1β over the level observed when treated with TNFα + IL-1β was also seen in chapter 3 (figure 3.8.3.2), when the Hec-1A cells were treated in the presence of RU486 (mifepristone), a progesterone antagonist. The reasons for this are unclear, but, it appears that there is some inhibition with the addition of TNFα to IL-1β, however, a direct inhibition on the expression of elafin in response to TNFα alone was not observed. Thus, it can therefore be assumed that there is some sort of interference in the synergistic actions of both these cytokines arising from the stromal conditioned media and this may merit further investigation. The overall increase in elafin expression in response to the inflammatory cytokines in the presence of stromal conditioned media is

suggestive of the presence of paracrine factor(s) from the stromal cells that may have a role in the up-regulation of elafin mRNA. The addition of progesterone to the media prior to the incubation with stromal cells was observed to reduce the expression of elafin mRNA (figure 4.3.1.2) when concurrently treated with TNFα (13-point) and TNFα + IL-1β (128-point; $P<0.001$), when compared to treatment in the presence of unconditioned progesterone supplemented media. As was observed for the previous conditioned media experiment (figure 4.3.1.2), the expression of elafin in response to the synergistic actions of TNFα + IL-1β was lower (22-point) than was observed when treated with IL-1β alone in the presence of stromal conditioned media (table 4.3.1.1). However, the inhibition of elafin mRNA in the presence of progesterone treated stromal conditioned media (figure 4.3.1.2) when compared to non-progesterone stromal conditioned media (figure 4.3.1.1 and table 4.3.1.1), supports the data obtained with menstrual phase endometrial explants (chapter 5), where elafin mRNA is maximal in biopsies obtained when circulating progesterone levels are low (King, Critchley et al. 2003). The endometrial explants are composed of a number of different cell types including stromal and epithelial cells (figure 4.1.1). Elafin has also been shown to be expressed by neutrophils which have also been observed to be present in the endometrium during menstruation (King, Critchley et al. 2003).

There may be some 'preparatory factor(s)' for the expression of elafin by epithelial cells that enables rapid expression upon exposure to inflammatory stimuli. An investigation was undertaken into the need for the inclusion of a 'spent' media control, which consisted of pre-conditioned epithelial media; replenished with 50%

fresh media (figure 4.1.1). These preliminary data (n=2) revealed that the Hec-1A cells that were exposed to epithelial conditioned media, expressed elafin more rapidly than unconditioned media and on average there was a slightly elevated level of elafin mRNA with conditioned media when the time points were averaged (figure 4.4.1; chart inset). The need for innate immune effectors to respond rapidly in the event of injury or infection may necessitate the need for 'ready to go' or 'on demand' mechanisms or factors. This would enable cells to perhaps mediate an interim response whilst the more timely preparation or infiltration of other innate or adaptive immune players.

Further consideration was given to the role of paracrine interactions and the role of epithelial signalling with respect to two-way signalling between the epithelial and stromal cells of the endometrium.

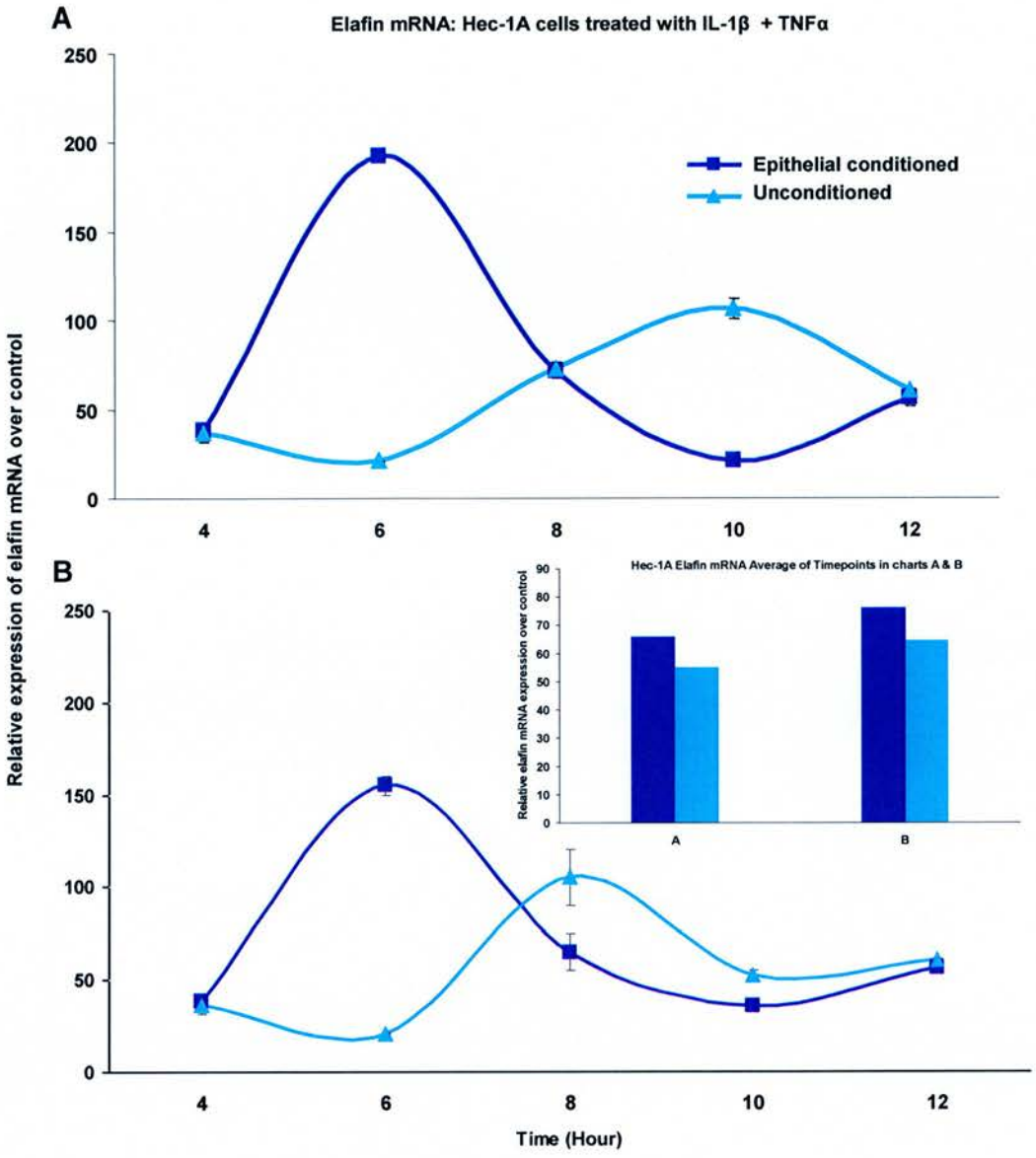


Figure 4.4.1 Elafin mRNA expression in the presence of pre-conditioned epithelial media (dark blue) and in unconditioned RPMI (light blue). The time courses were undertaken over 12 hour with 2 hourly sampling points (A and B). The bar chart inset shows the all the time point values averaged for A and B.

The use of a co-culture set-up was utilised and is described in section 4.2.1 and figure 4.2.1.1. The placement of the Hec-1A endometrial epithelial cell line with the

endometrial stromal cells (primary culture) and treatment with inflammatory cytokines TNFα and IL-1β allowed for the examination of epithelial-stromal interactions. Elafin mRNA expression by the Hec-1A cells was increased in the presence of stromal cells, and there was no change in the level of elafin mRNA expression in the stromal cells in the presence of Hec-1A cells (figure 4.3.2.1). This was in further support that there are stromal mediated factors involved in the up-regulation of elafin mRNA as was shown previously (figure 4.3.1.1). In chapter 3 it was also shown that progesterone was responsible for the up-regulation of elafin mRNA in response to inflammatory stimuli (figure 3.8.1.1). However, in the current chapter (figure 4.3.1.2), this effect was shown to be inhibited in the presence of stromal conditioned media. This was further demonstrated in the co-culture experiments (figure 4.3.2.2), where the presence of progesterone in co-culture inhibited the Hec-1A epithelial expression of elafin mRNA. This was in contrast with the increased expression observed with progesterone treated Hec-1A cells alone. There was no noticeable effect observed upon the expression of elafin by ESCs in mono or co-culture conditions. An investigation into the effect of treating the different components of the co-culture set-up was undertaken (figure 4.2.1.1). When the co-culture was treated in the bottom (stromal compartment), there was an increase in the level of elafin mRNA by both the ESCs and Hec-1A cells. The Hec-1A expression of elafin was greatest in response to treatment of the stromal cells with inflammatory cytokines (TNFα and IL-1β) compared with both Hec-1A cells alone and that where Hec-1A cells were treated directly in co-culture with ESCs. This may suggest that in the event of inflammation that greater urgency is attributed to the fact the stroma have received direct stimulation. This may be an indication of

the severity of an infection as it may be that the overlying epithelia may have been unable to address the initial threat or has been damaged in some way therefore allowing direct access to the underlying stroma (figure 4.1.1). An alternative explanation is that during the menstrual phase the functional layer of the endometrium (*Stratum functionalis*; figure 4.1.1), is shed, exposing the stroma to inflammatory mediators (Critchley, Kelly et al. 2006). This is also a period of increased vulnerability for the endometrium and perhaps this necessitates the need for expression of elafin by the stromal cells. This may also suggest an explanation for the high level of elafin mRNA observed in endometrial explants collected from the menstrual phase of the cycle (chapter 5; and (King, Critchley et al. 2003). However, the stromal expression of elafin protein was not shown during the menstrual phase, but, was localised to the neutrophil population (King, Critchley et al. 2003).

Preliminary analyses of elafin protein within the co-culture set-up (figures 4.3.3.1 to 4.3.3.3), revealed some interesting data and this merits further investigation. The main observation was the consistent differences in the level of elafin protein that could be detected within the co-culture wells. The different components of the well are separated by a thin membrane designed to allow the exchange of cell signalling molecules. The protein data demonstrates that there is very little mixing of the culture media between the two compartments.

In summary, the endometrium is made up of a number of different cell types and these cells are involved in cell-cell communication and thus, a number of paracrine factors need to be considered. In the current chapter it has been shown that there are stromal mediated factors which influence the expression of elafin by epithelial cells (Hec-1A) and this is summarised in figure 4.4.2. An increase in elafin mRNA was observed in the presence of stromal derived media or in the presence of stromal cells in co-culture. It was further demonstrated that the effect of progesterone upon the epithelial expression of elafin mRNA may be mediated via the stromal cells. The treatment of Hec-1A cells with progesterone along with inflammatory stimuli increases elafin expression (chapter 3 and 4), however, the presence of stromal cells or stromal derived media, elafin mRNA was inhibited. Elafin mRNA has been shown to be maximally expressed in the endometrium during the menstrual phase when circulating levels of progesterone are low in chapter 5 (figure 3.8.1.1) and in previous studies (King, Critchley et al. 2003). This is suggestive that the effect of progesterone upon the epithelial expression of elafin is indirect and may be as a consequence of progesterone mediated effects upon the stroma which in turn exhibits an effect upon the epithelium. Progesterone has been shown to stimulate a number of factors from the stromal cells which may act upon the epithelial cells of the endometrium. It may be that progesterone acts to produce an inhibitor of elafin expression via the stromal cells. It was demonstrated in chapter 3 that the cytokine TGFβ-1 caused a decrease in the expression of elafin in Hec-1A cells treated with inflammatory stimuli (figures 3.4.3.1 and 3.4.3.2). The inhibition of SLPI has also been reported in response to TGFβ-1 (Jaumann, Elssner et al. 2000). In the endometrium it has been demonstrated that progesterone increases stromal

expression of TGFβ (Bruner, Rodgers et al. 1995). Thus, it may be that the progesterone mediated expression of TGFβ-1 by the stroma is responsible for the inhibition of elafin mRNA in menstrual endometrium (King, Critchley et al. 2003), and in the presence of stromal cells or stromal derived media with progesterone. The stromal mediated expression of TGFβ-1 in response to progesterone has also been shown to inhibit the epithelial expression of the matrix metalloproteinase, MMP-7 (matrilysin) (Bruner, Rodgers et al. 1995). MMP-7 is co-expressed with α-defensins in the paneth cells of the murine gut, and has the ability to cleave pro-α-defensins into the active and mature form (Wilson, Ouellette et al. 1999). Elafin is also expressed as a pro-form, trappin-2 (Guyot, Zani et al. 2005) and investigations in the lung have identified tryptase as a candidate elafin-releasing enzyme (Guyot, Zani et al. 2005). Thus, the role of MMP-7 in endometrial expression of natural antimicrobials including elafin was investigated further within this thesis (chapter 5-8).

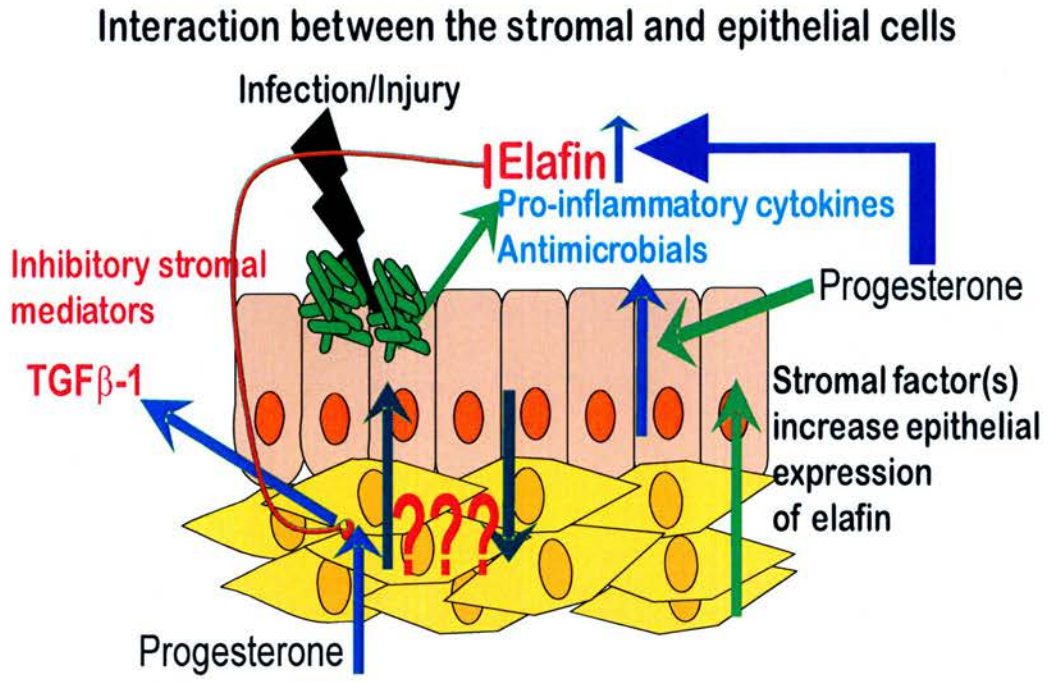


Figure 4.4.2 Progesterone was shown to up-regulate elafin mRNA in Hec-1A cells in response to inflammatory stimuli (chapter 3). In the presence of stromal cells or stromal derived media, the Hec-1A expression of elafin mRNA is also increased (green arrows). However, this expression was inhibited (red) in the presence of the stroma, suggesting the presence stromal factors with influence over the epithelial expression of elafin. Also in chapter 3 it was shown that TGFβ-1 inhibited the inflammatory expression of elafin mRNA. Progesterone up-regulates stromal expression of TGFβ-1 and this may be one of the paracrine factors involved in the epithelial expression of elafin. However, there are stromal derived factors in the absence of progesterone that are responsible for the increase in elafin mRNA. There are also other candidates including MMP-7 that are progesterone/stromal mediated that may have a role.

4.4.1 Future work

The preliminary data contained within this chapter demonstrates a likely role for paracrine mediated factor(s) in the expression of elafin. The cytokine TGFβ-1 is a candidate factor for this stromal-epithelial interaction, and was shown to inhibit elafin expression in the Hec-1A cell line and is documented to be upregulated in response to progesterone. The analyses of stromal derived culture media for the presence of TGFβ-1 both in response to progesterone and/or the presence of epithelial cells would help confirm this interaction. There are many commercial ELISA kits available for the examination of TGFβ-1 production and would be useful in this case. Further studies involving the inhibition or blocking of TGFβ-1 expression and the examination on the effects of elafin expression would also be informative. It was also identified from the literature that MMP-7 is downregulated via progesterone mediated stromal expression of TGFβ-1 (Bruner, Rodgers et al. 1995). Furthermore, there is a possibility of a role for MMP-7 in the splicing of pre-formed antimicrobials in the endometrium. An analysis of the effect of MMP-7 upon the pre-mature forms of antimicrobials such as the β-defensins and elafin would be informative along with a measurement of expression levels in co-culture experiments. The significance of MMP-7 expression is addressed through the remainder of this thesis and discussed in chapters 5 to 8.

There have also been a number of inconsistencies observed with regard the hormonal influence over the expression of some antimicrobials. In the endometrium it has been shown that hBD1 levels are maximal during the high progesterone mediated stages of the menstrual cycle (Fleming, King et al. 2003). However, *in vitro* studies

showed that hBD1 mRNA was suppressed in the presence of progesterone, indicative of an indirect or coincident effect. Thus, further work would examine the role of stromal-epithelial interaction upon the expression of other natural antimicrobials such as the β-defensins and identification of any factors or mechanisms.

Decidualised stromal cells in combined culture with the epithelial cell line upon the expression of natural antimicrobials and the presence of different factors or interactions would also be worth investigating. The use of decidualised stromal cells may provide a mechanism for the further understanding of both the expression and regulation of natural antimicrobials during pregnancy.

Chapter 5:

A comparative analysis of elafin, SLPI, TGFβ-1 and MMP-7 mRNA expression in pregnant and non-pregnant endometrium.

Chapter 5: A comparative analysis of elafin, SLPI, TGFβ-1 and MMP-7 mRNA expression in pregnant and non-pregnant endometrium.

5.1 Introduction

The endometrium undergoes morphological changes in response to the interchanging balance of a number of mediators. These changes are under the control of the steroid hormones, oestradiol and progesterone. It has been shown that many of the factors thought to be involved in the modulation of the immune defence of the female reproductive tract are similarly affected by hormone levels (Wira and Fahey 2004; Schaefer, Fahey et al. 2005; Wira, Fahey et al. 2005; Wira, Grant-Tschudy et al. 2005). Cytokines and natural antimicrobials are amongst the molecules which undergo cyclical changes in expression. It has been previously shown that both elafin and SLPI expression is differential across the menstrual cycle. SLPI has been shown to be upregulated during the mid secretory stage of the menstrual cycle where circulatory progesterone levels are maximal (King, Critchley et al. 2003). It has further been demonstrated that *in vitro* treatment of the breast cancer cell line T47D with progesterone gives rise to an increase in SLPI mRNA (King, Morgan et al. 2003). It has been shown that the endometrial epithelial cell line (Hec-1A), also demonstrates an increase in SLPI when treated with progesterone, and that this can be reversed with the addition of the progestin antagonist RU486 (mifepristone), (chapter 3). Elafin has been shown to be expressed during the late secretory and maximally during the menstrual phase when progesterone levels are low (King, Critchley et al. 2003; King, Morgan et al. 2003). The data presented in chapter 3, have shown elafin to be increased in response to progesterone treatment of Hec-1A

cells. A further increase in elafin mRNA was observed when the cells were treated with the progesterone receptor antagonist, RU486 (mifepristone), which was suggestive of an increase in elafin being brought about by the act of progesterone withdrawal (King, Morgan et al. 2003), and this observation is consistent with the increase observed in late secretory endometrium. In chapter 4 it has been demonstrated that the effect of progesterone on elafin mRNA expression is mediated by paracrine factors, such as TGFβ-1 and MMP-7. The addition of progesterone to Hec-1A cells in the presence of endometrial stromal cells resulted in an inhibition of expression in response to inflammatory mediators. Possible candidates for this indirect effect of progesterone remain to be established. TGFβ-1 has been shown to inhibit elafin expression (chapter 3). TGFβ-1 has been shown to increase in the presence of progesterone and is expressed mainly by the endometrial stromal cells (Bruner, Rodgers et al. 1995). The data presented in this thesis are suggestive of an association between MMP7 (matrilysin) and elafin (chapters 6-8). MMP-7 has also been shown to be mediated by progesterone concentration and may act in a paracrine fashion between the endometrial stromal and epithelial cells (Rodgers, Osteen et al. 1993; Osteen, Rodgers et al. 1994; Bruner, Rodgers et al. 1995; Osteen, Keller et al. 1999).

Thus, it was deemed necessary to re-examine the expression of these proteins in a comparative fashion in primary endometrial explants in order to further explore the significance of the cell culture data presented in Chapters 3 and 4.

5.2 Materials and methods

5.2.1 Human uterine tissue collection

Endometrial (n=14; table 5.2.1.1) and decidual (n=5; 5.2.1.2) samples were collected as detailed in chapter 2 (section 2.1). The endometrial samples provided for study in this chapter were in the form of extracted and validated RNA cDNA from the laboratory archive (Prof Critchley group; LREC 1994/6/17) and was prepared in a similar manner to that described in section 2.3 (chapter 2). The decidual samples (LREC 04/S1103/20), were prepared for RNA analysis as described in section 2.3.

Sample No.	Age	Parity	Endometrial histological stage at time of biopsy	Blood E2 (pmol/l)	Blood P4 (nmol/l)
1	42	2+0	Menstrual	167	1.6
2	39	1+0	Menstrual*	118	2.94
3	38	2+0	Menstrual*	151	4.31
4	25	1+0	Proliferative	895	2.5
5	41	3+3	Proliferative	842	7.1
6	32	1+0	Proliferative	953	3.7
7	39	2+0	Early Secretory	604	42.5
8	45	0+0	Early Secretory	680	106.25
9	44	0+1	Mid Secretory	276	42.1
10	40	3+0	Mid Secretory	490	22.9
11	44	2+0	Late Secretory	602	14.7
12	44	2+0	Late Secretory	183	5.4
13	42	2+0	Late Secretory	129	5.7
14	44	3+0	Late Secretory*	423	5.19

Table 5.2.1.1. Details of the endometrial samples used when analysing the expression of elafin, SLPI, MMP-7 and TGFβ-1 across the menstrual cycle. Three of the samples were undetermined upon histological analysis and have been staged according to the patients last menstrual period (LMP) and sex steroid levels at the time of biopsy (*).

Sample No.	Age	Group	Parity	Gestation	Procedure
1	33	2	3+3	10+5	Surgical Termination of Pregnancy
2	28	2	0+0	10	Surgical Termination of Pregnancy
3	22	2	1+1	8+6	Surgical Termination of Pregnancy
4	24	2	0+0	10	Surgical Termination of Pregnancy
5	29	2	1+3	10+6	Surgical Termination of Pregnancy

Table 5.2.1.2. Details of first trimester decidual samples used when analysing the expression of elafin, SLPI, MMP-7 and TGFβ-1. Group 2 contained samples of decidua collected from women undergoing termination of pregnancy.

5.2.2 QRT-PCR

Elafin, SLPI, TGFβ-1 and MMP-7 expression was determined using quantitative real time PCR as detailed in chapter 2.

5.2.3 Statistical Analysis

The PCR results in this chapter were analysed by ANOVA for significant difference. Fisher's protected least significant difference (PLSD) was used to assign individual differences (PRISM).

5.3 Results

Endometrial tissue analyses

5.3.1 Elafin mRNA expression in endometrium

Elafin mRNA expression was maximal in the menstrual phase biopsies (figure 5.3.1.1; Menstrual/proliferative, menstrual/early secretory and menstrual/mid-secretory $P<0.001$; menstrual/late secretory $P<0.01$).

5.3.2 SLPI mRNA expression in endometrium

SLPI mRNA levels were maximally increased in mid-secretory endometrium (figure 5.3.2.1; mid-secretory/proliferative $P<0.01$; mid-secretory/early secretory $P<0.05$). SLPI mRNA was increased in late secretory phase endometrium (figure 5.3.2.1; late secretory/proliferative and late secretory/early secretory $P<0.05$) and in menstrual phase endometrium (figure 5.3.2.1; menstrual/proliferative and menstrual/early secretory $P<0.05$).

5.3.3 MMP-7 mRNA expression in endometrium

MMP-7 mRNA levels were found to be maximal in menstrual endometrium (figure 5.3.3.1; menstrual/early secretory, menstrual/mid-secretory and menstrual/late secretory $P<0.01$). In addition there was an increase in the level of MMP-7 mRNA in proliferative phase endometrium (figure 5.3.3.1; not statistically significant)

5.3.4 TGFβ-1 mRNA expression in endometrium

TGFβ-1 mRNA expression was found to be present across the menstrual cycle and did not demonstrate any significant variation in any of the menstrual phases.

5.3.5 Expression of elafin, SLPI, MMP-7 and TGFβ-1 in decidua

First trimester decidual biopsies (n=5) were examined for the expression of elafin (blue), SLPI (green), MMP-7 (yellow) and TGFβ-1 (purple) mRNA (figure 5.3.5.1). All genes were shown to be expressed and no significant differences were demonstrated.

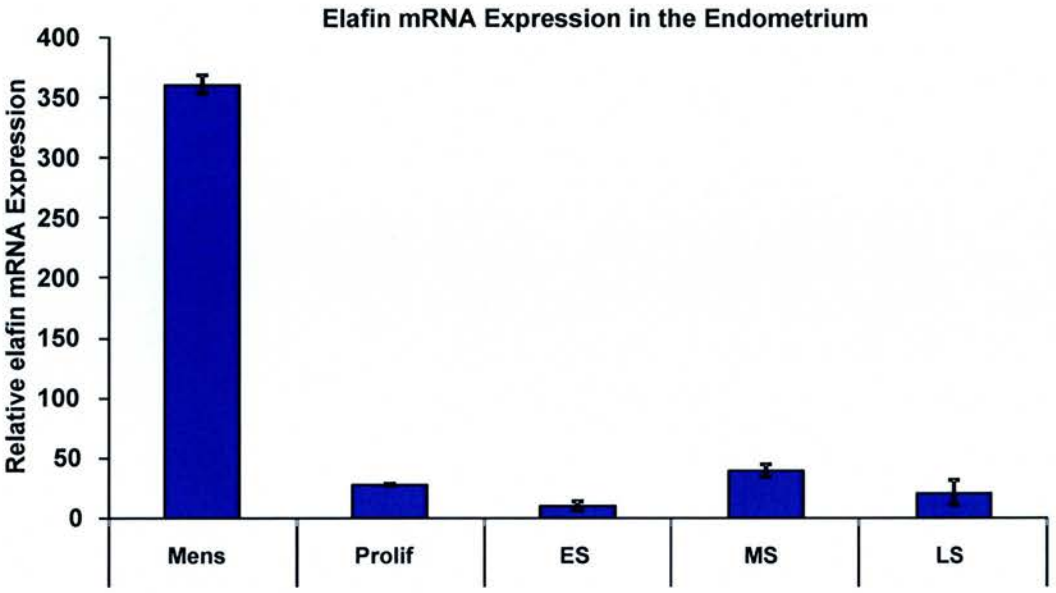


Figure 5.3.1.1. Elafin mRNA expression within human endometrium across the menstrual cycle (n= detailed within the x axis). Data are presented as relative changes in mRNA expression relative to a control (negative control obtained from a sample of early secretory endometrium), given a nominal value of 1, mean ± s.e.m. Mens/prolif, Mens/ES and Mens/MS $P<0.001$; Mens/LS $P<0.01$.

Abbreviations: Mens, Menstrual phase; Prolif, proliferative phase; ES, early secretory phase; MS, mid secretory phase; LS, late secretory phase.

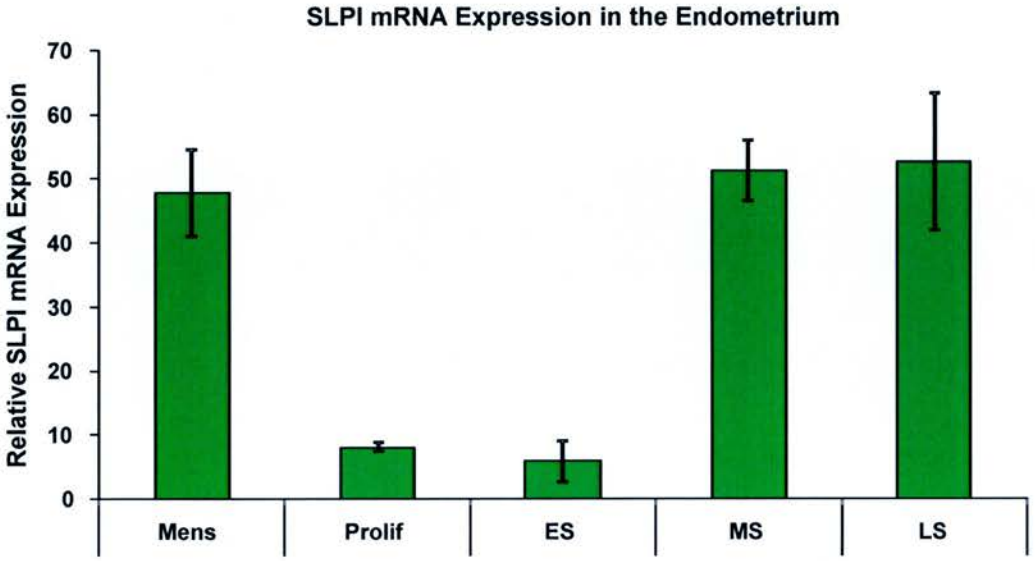


Figure 5.3.2.1. SLPI mRNA expression within human endometrium across the menstrual cycle (n= detailed within the x axis). Data are presented as relative changes in mRNA expression relative to a control (negative control obtained from a sample of early secretory endometrium), given a nominal value of 1, mean ± s.e.m. Mens/prolif, Mens/ES and Mens/MS $P<0.001$.

Abbreviations: Mens, Menstrual phase; Prolif, proliferative phase; ES, early secretory phase; MS, mid secretory phase; LS, late secretory phase.

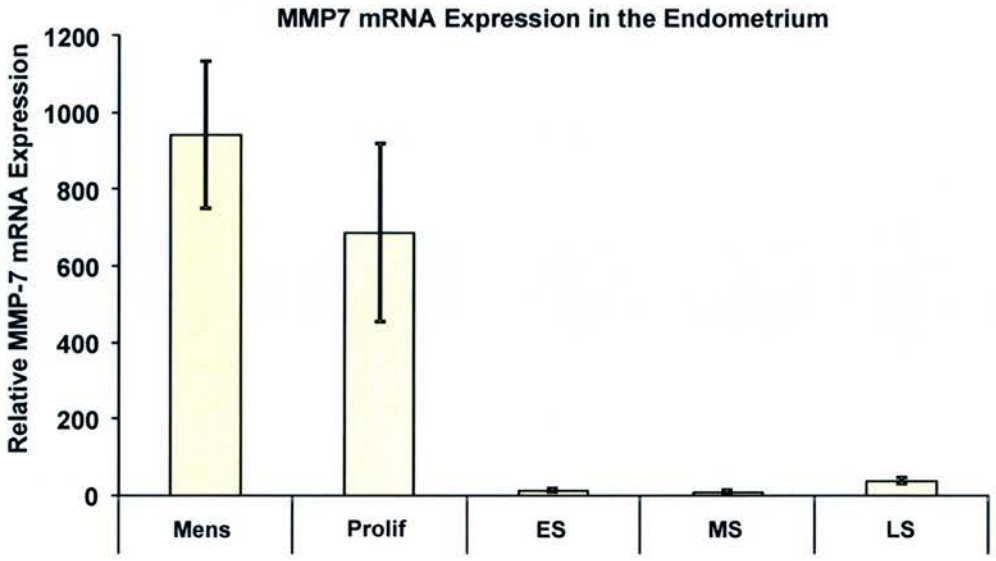


Figure 5.3.3.1. MMP-7 mRNA expression within human endometrium across the menstrual cycle (n= detailed within the x axis). Data are presented as relative changes in mRNA expression relative to a control (negative control obtained from a sample of mid secretory endometrium), given a nominal value of 1, mean ± s.e.m. Mens/ES, Mens/MS and Mens/LS $P<0.001$.

Abbreviations: Mens, Menstrual phase; Prolif, proliferative phase; ES, early secretory phase; MS, mid secretory phase; LS, late secretory phase.

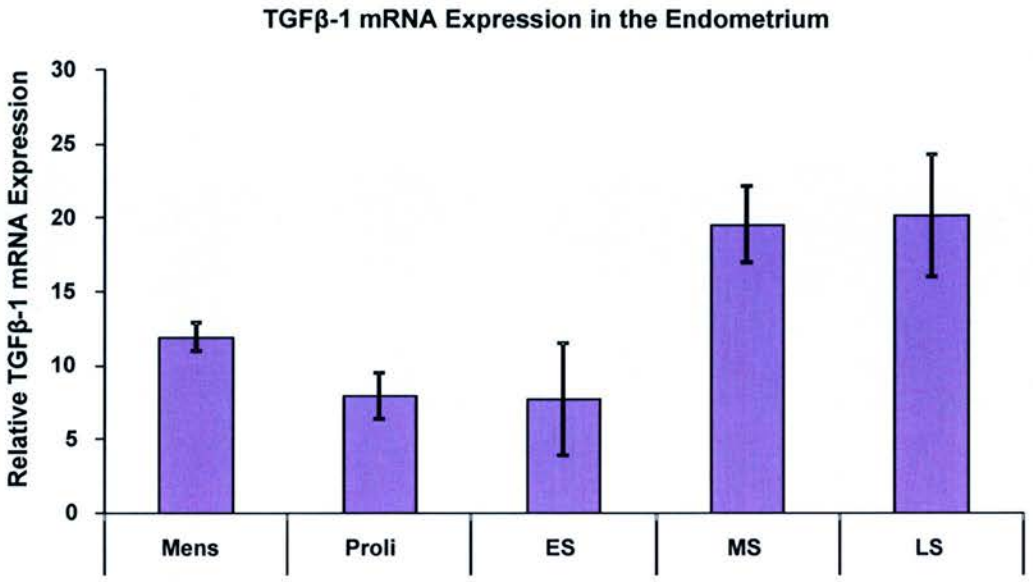


Figure 5.3.4.1. TGFβ-1 mRNA expression within human endometrium across the menstrual cycle (n= detailed within the x axis). Data are presented as relative changes in mRNA expression relative to a control (negative control obtained from a sample of early secretory endometrium), given a nominal value of 1, mean ± s.e.m. Abbreviations: Mens, Menstrual phase; Prolif, proliferative phase; ES, early secretory phase; MS, mid secretory phase; LS, late secretory phase.

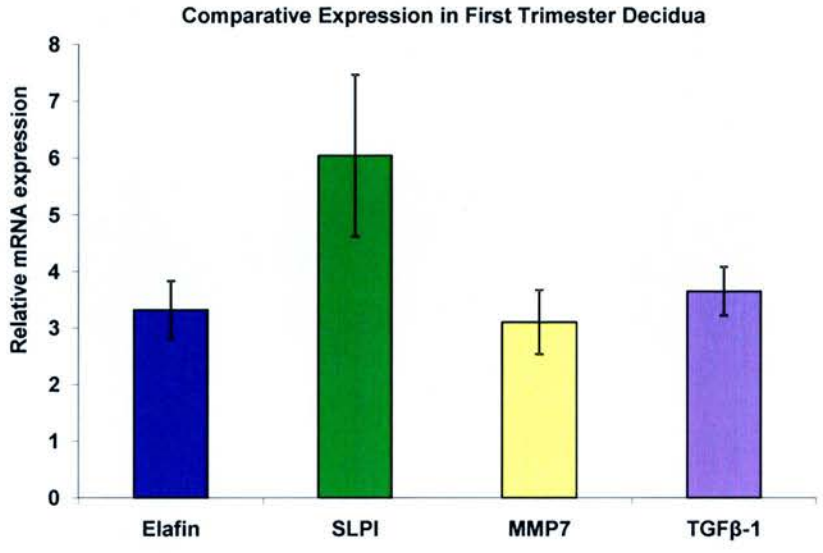


Figure 5.3.5.1 Elafin (blue), SLPI (green), MMP-7 (yellow) and TGFβ-1 (lilac) mRNA expression in first trimester decidual biopsies (n=6). Data are presented as relative changes in mRNA expression relative to a control (negative control obtained from a sample of early secretory endometrium), given a nominal value of 1, mean ± s.e.m.

5.4 Discussion

The role of the steroid hormones in the mediation of the innate immune response of the female reproductive tract has been previously documented (Critchley, Kelly et al. 2001; Kayisli, Guzeloglu-Kayisli et al. 2004; Herath, Fischer et al. 2006). This interaction may be direct or indirect via intermediate factor(s). Progesterone and oestradiol have been shown to be involved with the differential expression of cytokines throughout the menstrual cycle (Polli, Bulletti et al. 1996; Yang, Chen et al. 1996; Agarwal and Marshall 1999; Verthelyi and Klinman 2000; Chegini, Ma et al. 2002; Roberts, Luo et al. 2005). This effect may result from a change in the balance or equilibrium that exists between progesterone and oestradiol. However, it has been proposed that the act of withdrawal of either steroid may also stimulate a response. The natural antimicrobials have been previously shown to be regulated by the cyclical changes of steroid hormone levels (Quayle, Porter et al. 1998; King, Critchley et al. 2003; King, Fleming et al. 2003; King, Morgan et al. 2003). This regulation is believed to provide the female reproductive tract with a broad level of coverage, with the differential expression of the proteins across the menstrual cycle.

In the female reproductive tract it has been shown that elafin mRNA is increased in late secretory endometrium coincident with the withdrawal of progesterone, and maximally expressed in menstrual endometrium when circulating concentrations of progesterone are low (King, Critchley et al. 2003; King, Morgan et al. 2003). The endometrial samples collected across the menstrual cycle, investigated within this chapter confirm these observations (figure 5.3.1.1). The withdrawal of progesterone

has been suggested to be stimulatory to an inflammatory response, with the up-regulation of pro-inflammatory mediators (Stites and Siiteri 1983; Critchley, Kelly et al. 2001; King, Critchley et al. 2001). Thus, it may be this indirect response which mediates the increase in elafin expression in late secretory endometrium. However, it has also been shown that leukocytes which infiltrate the late secretory/perimenstrual endometrium, also express elafin (King, Critchley et al. 2003). Thus, this may account for the raised levels of elafin mRNA. It is possible that progesterone has an inhibitory role on factors which serve to inhibit elafin, and that in the absence of progesterone this inhibition is ablated. It has been demonstrated that the expression of TGFβ-1 is increased in endometrial stromal cells in response to progesterone (Polli, Bulletti et al. 1996; Lim, Odukoya et al. 1998). Data described in the present thesis demonstrated that the *in vitro* treatment of an epithelial cell line with TGFβ-1, resulted in a decrease in elafin mRNA (chapter 3). Although not statistically significant there does also appear to be an increase in the level of TGFβ-1 mRNA in the mid and late secretory endometrium in comparison with proliferative and early secretory endometrium (figure 5.3.4.1). This is consistent with an up regulation in the presence of progesterone and it is possible that this in turn serves to have an inhibitory role over elafin.

SLPI mRNA levels are shown to be increased in mid and late secretory endometrium (figure 5.3.2.1) and this is in support of previous descriptions of SLPI up-regulation in response to progesterone (King, Morgan et al. 2003). It has also been shown in this thesis that SLPI may have an inhibitory effect upon the expression of elafin (chapter 8). Perhaps SLPI acts as an indirect mediator in the progesterone dependent

decrease in elafin mRNA *in vivo*. However, the co-culture studies (chapter 4) are suggestive of paracrine mediated factor(s), as the inhibition of elafin in the presence of progesterone and stromal cells represents that observed in primary endometrial samples. There is also an increase in SLPI mRNA in menstrual endometrium, which was not expected based on previous work (King, Morgan et al. 2003). The present study contained a smaller number of subjects, which may be an explanation for the results obtained that differ with previous larger studies (King, Critchley et al. 2000; Fleming, King et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003).

It was identified in the present thesis that MMP-7 has an associative relationship with elafin, and this has been investigated in endometrial biopsies obtained across the menstrual cycle. It has been previously shown, that MMP-7 is expressed in late secretory, menstrual and proliferative endometrium and has not been detected in early or mid secretory endometrium (Rodgers, Osteen et al. 1993). The present study has demonstrated that MMP-7 mRNA expression is increased in menstrual and proliferative endometrium, coinciding with both reduced progesterone and increasing oestradiol levels (figure 5.3.3.1). MMP-7 has been shown to be inhibited in the presence of progesterone (Bruner, Rodgers et al. 1995; Osteen, Sierra-Rivera et al. 1997; Osteen, Keller et al. 1999), and in studies described within this thesis (chapters 6-8). The present study was undertaken to investigate MMP-7 as a possible candidate for the processing of pre-elafin (trappin-2) into the mature form (elafin) or alternatively may function to degrade elafin. Timecourse studies in cell culture have shown a similar pattern of MMP-7 to that exhibited by elafin mRNA, and in an apparent lag i.e. MMP-7 mRNA expression appears to follow that of elafin (figure

8.1.1, chapter 8). In the endometrium, elafin is expressed in late secretory and in menstrual phase samples – whilst MMP-7 is observed to be increased in the menstrual and proliferative samples. This would be in support of the 'lag relationship' in expression. TGFβ-1 reduces the expression of epithelial MMP-7, and with higher circulatory levels of progesterone there is an increase in the level of TGFβ-1. Thus, this may represent an indirect effect of progesterone via TGFβ-1.

Elafin, SLPI, MMP-7 and TGFβ-1 have been shown to be expressed in first trimester decidua. There is no significant difference observed between these proteins in the decidual samples that were studied, however, there is almost 3-point greater expression of SLPI mRNA. Progesterone levels are maintained in decidua, and it has been previously shown that SLPI levels are increased during the first trimester of pregnancy (King, Fleming et al. 2002; King, Critchley et al. 2003; King, Morgan et al. 2003). Further investigation of the role and possible interaction of these molecules is addressed elsewhere in the present thesis (Chapters 6-8).

In summary, the investigations undertaken in the present chapter were in response to observations throughout the current thesis that a relationship likely exists between the expression of SLPI, elafin and MMP-7 mRNA. Furthermore, the role of TGFβ-1 upon the expression of these molecules and as a possible mediator was examined. The expression of elafin and SLPI in the endometrium has been previously shown to be governed by the cyclical changes of the sex steroids across the menstrual cycle (King, Critchley et al. 2003). However, the expression of elafin and SLPI in relation to other innate immune mediators has not been considered previously. In chapter 6 and 7 of the current thesis an associative expression between both SLPI and elafin with MMP-7 has been observed. Whilst in chapter 8 the direct addition of SLPI has been shown to decrease the Hec-1A expression of both elafin and MMP-7 mRNA. The observations and questions raised in this current chapter are summarised in figure 5.4.1. In this chapter it is shown that MMP-7 mRNA is expressed with elafin during the menstrual phase and is expressed in the proliferative phase immediately following. This may be to allow the enzyme to degrade elafin or to process elafin and it could also be entirely coincidental. The expression of elafin and MMP-7 in the menstrual phase may suggest an involvement in the breakdown of the functional layer, with anti-protease activity. Whilst the expression of MMP-7 during the proliferative phase may indicate a role in the rebuilding of the functional layer and may also suggest the breakdown of molecules such as elafin following the high levels of expression during the menstrual phase. The demonstration that TGFβ-1 expression is highest during the secretory phase may serve as evidence of the presence of progesterone mediated inhibitors of elafin and MMP-7. It has also been reported previously that endometrial stromal cells upregulate the expression of

TGFβ-1 in response to progesterone (Polli, Bulletti et al. 1996). The increased levels of SLPI mRNA during the secretory phase may also inhibit the expression of elafin and MMP-7 as was shown in chapter 8. The data contained within the current chapter served to both lend support to observations elsewhere as discussed and to raise further questions.

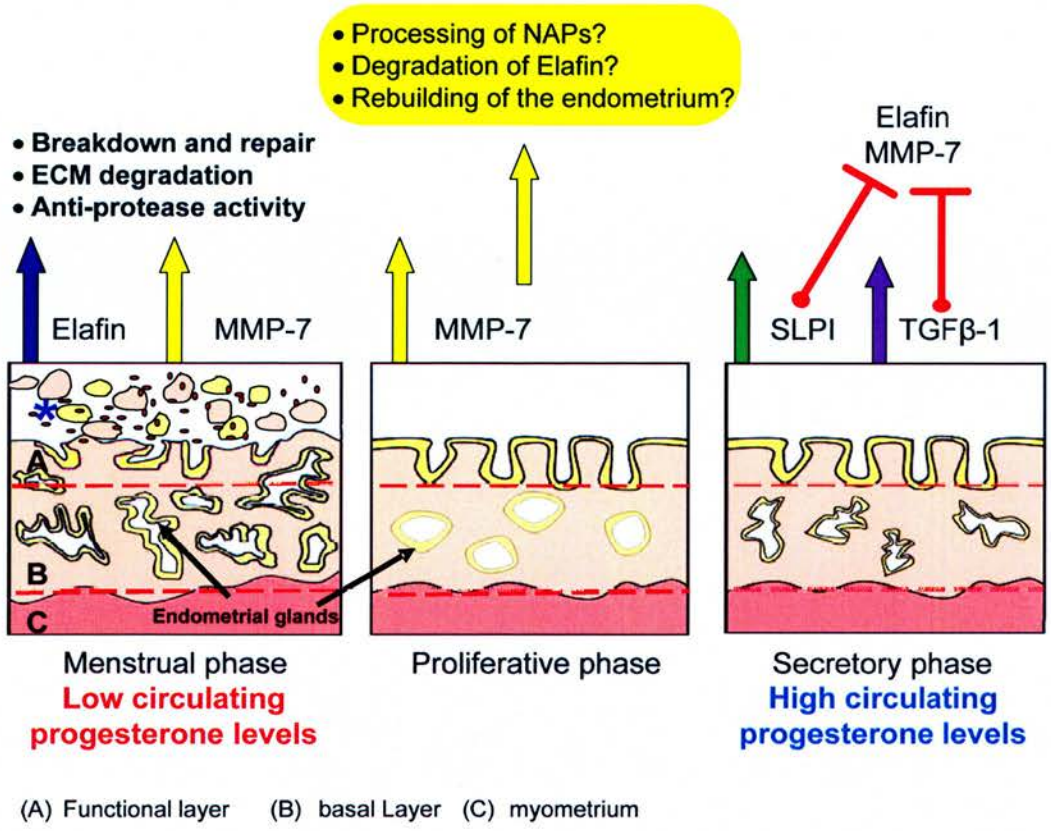


Figure 5.4.1 Schematic summary of the differential expression of SLPI (green), elafin (blue), MMP-7 (yellow) and TGFβ-1 (purple) at different stages of the menstrual cycle. During the menstrual phase, involving tissue breakdown (*) and when circulating levels of progesterone are low, elafin and MMP-7 have been shown to be highly expressed. During the proliferative phase, MMP-7 is expressed at similar levels to those observed in the menstrual phase. In mid secretory phase when circulating progesterone levels are high, SLPI is maximally expressed. The highest level of TGFβ-1 is also observed in the secretory phase. TGFβ-1 and SLPI have been shown to be upregulated by progesterone and inhibitory towards the expression of elafin and MMP-7 mRNA.

Chapter 6:

Natural antimicrobial expression in human Fallopian tube with and without the presence of an ectopic pregnancy.

Chapter 6: Natural Antimicrobial Expression in human Fallopian tube with and without the presence of an ectopic pregnancy

6.1 Introduction

Ectopic pregnancy is when the gestational tissues are located out with the uterine cavity. In the UK ectopic pregnancy remains a cause of maternal death (RCOG 2004; Corpa 2006). Tubular pregnancies result from the mechanical failure of the fertilised oocyte being delivered to the uterus in a timely fashion. The precise underlying causes of an ectopic pregnancy are poorly understood. It has been proposed that damage to the Fallopian tube due to sexually transmitted infections such as chlamydia and gonorrhoea, may be a cause of ectopic gestation (Ankum, Mol et al. 1996; Job-Spira, Fernandez et al. 1999). Chlamydia has been a well characterised causal agent of chronic inflammation leading to pelvic inflammatory disease (PID) (Bouyer, Coste et al. 2003). The prevalence of ectopic pregnancy has increased over the past two decades and this has been linked to the increase in PID (Westrom, Joesoef et al. 1992; Bouyer, Coste et al. 2003). It has been reported that women who present with an ectopic pregnancy are found to have high levels of antibodies to chlamydia (Svensson, Mardh et al. 1985; Chow, Yonekura et al. 1990; Kihlstrom, Lindgren et al. 1990; Odland, Anestad et al. 1993). There is a 2-fold greater risk of an ectopic pregnancy in women with a current or past history infection with chlamydia (Westrom, Joesoef et al. 1992). There is a 4-fold risk of an ectopic pregnancy for woman with a tubal pathology such as salpingitis, which involves damage to the endosalpingeal mucosa (Westrom, Joesoef et al. 1992). This damage

may result in obstruction of the migrating embryo, giving rise to an ectopic implantation (Job-Spira, Fernandez et al. 1999; Bouyer, Coste et al. 2003).

A number of molecular factors and proteins have also been implicated in the regulation of implantation and irregularities in these factors may be involved in ectopic pregnancy. The possible inflammatory role in the pathogenesis of an ectopic pregnancy gives rise to an interest in the role of immune effectors such as natural antimicrobials in both the normal and ectopic implantation.

Currently very little is known about the expression or role of natural antimicrobials within the Fallopian tubes. Natural antimicrobial expression in the Fallopian tube has been previously described for human defensin 5 (HD5), a member of the α-defensins (Quayle, Porter et al. 1998), and SLPI (Ota, Shimoya et al. 2002). In contrast the expression levels of natural antimicrobials in the pregnant and non-pregnant endometrium has been well described and data have been presented in an earlier chapter in this thesis (chapter 5), (Quayle, Porter et al. 1998; King, Fleming et al. 2002; King, Critchley et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003; Wira, Fahey et al. 2005). The role of antimicrobials in the decidua of failed pregnancies such as in the event of miscarriage or a tubular ectopic pregnancy has not been described and will be addressed in the next chapter. The expression of natural antimicrobials in the endometrium has also been shown to be differentially expressed across the menstrual cycle in response to changes in circulating sex steroid hormones (King, Fleming et al. 2003). This differential expression in response to sex steroids, has been further supported with *in vitro* studies using cell lines from the

cervix, breast (King, Morgan et al. 2003) and in an endometrial cell line (data described in chapter 3).

This study aimed to investigate the expression of natural antimicrobials in the normal Fallopian tube and in samples that were collected at the time of surgical management of ectopic pregnancy. The expression patterns of natural antimicrobials in normal Fallopian tube samples were compared with endometrial tissue samples that had been accurately categorised according to menstrual cycle phase. The Fallopian tube samples obtained from the surgical management of ectopic pregnancy were investigated for differential natural antimicrobial expression and compared with normal Fallopian tube biopsies. The emerging pattern that has been observed between the expression patterns of SLPI, elafin and MMP-7 was further explored.

6.2 Materials and Methods

6.2.1 Sample collection

Fallopian tube biopsies (n=10) were collected from fertile woman undergoing hysterectomy procedures for benign indications (age 18-45 years). Fallopian tube biopsies were utilised for investigations into the expression of natural antimicrobials. Fallopian tube biopsies were classified according to phase of ovarian/menstrual cycle (n=7) as detailed in table 6.2.1.1. The samples were dated at the time of biopsy using serum levels of E_2 and P_4 (figure 6.2.1.1) and histological dating of the corresponding endometrial samples.

Fallopian tube biopsies (n=9) were collected from women undergoing surgical management of ectopic pregnancy (age 25-40 years). The details of these samples are provided in table 6.2.1.2.

Tissue Sample	Age	Parity	Endometrial histological stage at time of biopsy	Blood E_2 (pmol/l)	Blood P_4 (nmol/l)	Surgery
1	37	2+0	Late secretory	392.18	3.06	Total abdominal hysterectomy
2	41	2+0	Proliferative	1022.87	0.81	Total abdominal hysterectomy
3	37	2+2	Proliferative	940.44	3.82	Total abdominal hysterectomy
4	44	3+1	Proliferative	829.42	4.19	Total abdominal hysterectomy
5	44	2+2	Proliferative	116.3	2.88	Sub-total abdominal hysterectomy
6	36	3+0	Proliferative	770.63	5.16	Total abdominal hysterectomy
7	32	1+0	Mid secretory	549.91	88	Total abdominal hysterectomy

Table 6.2.1.1 Details of the Fallopian tube samples used when analysing natural antimicrobial expression across the menstrual cycle.

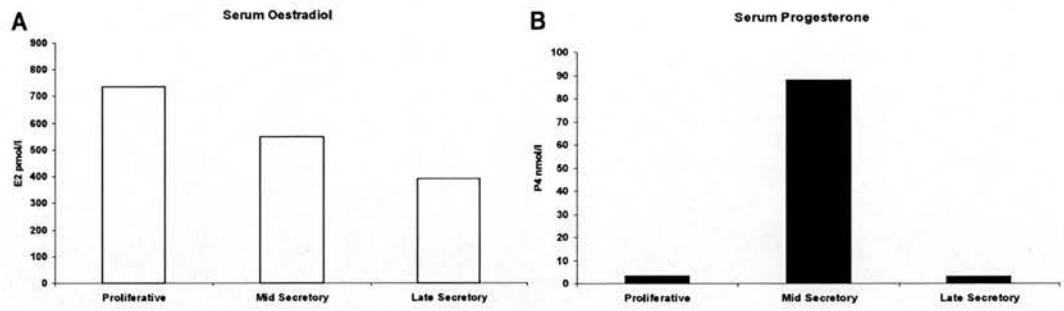


Figure 6.2.1.1 Graphical demonstration of serum oestradiol (A) and progesterone (B) levels in peripheral blood obtained at the time of biopsy for the Fallopian tubes investigated for cyclic expression of natural antimicrobials.

Tissue Sample	Age	Parity	Gestation	Procedure
1	30	0+0	6+2	Laparoscopic salpingectomy
2	25	1+1	7+1	Laparoscopic salpingectomy
3	40	1+0	8+3	Laparoscopic salpingectomy
4	31	2+1	5+4	Laparoscopic salpingectomy
5	28	2+2	5+3	Laparoscopic salpingectomy
6	26	0+0	6+0	Laparoscopic salpingectomy
7	26	1+0	6+6	Laparoscopic salpingectomy
9	33	0+0	11+1	Laparoscopic salpingectomy

Table 6.2.1.2 Details of the Fallopian tube samples obtained from women with an ectopic gestation (tubal pregnancy).

6.2.2 RNA extraction and PCR

RNA was extracted from frozen biopsy samples (Fallopian) stored in RNA later (Ambion), using the Qiagen proteinase K protocol and cDNA prepared, as detailed in

section 2.3, chapter 2. Expression levels of SLPI, Elafin, MMP-7 and hBD1-4 mRNA were determined using quantitative real time PCR (as detailed in section 2.3.3, chapter 2).

6.2.3 Immunohistochemistry

Elafin and SLPI protein was localised in the Fallopian tube using the protocols described in section 2.6, chapter 2.

6.2.4 Statistical Analysis

The PCR results in this chapter were analysed by ANOVA for significant difference. Fisher's protected least significant difference (PLSD) was used to assign individual differences (PRISM).

Statistical analysis was not carried out on cyclic Fallopian biopsies due to the low number of clearly endometrially staged samples.

6.3 Results

6.3.1 Differential expression of antimicrobials and MMP-7 in relation to the stage of ovarian cycle as determined by histological dating of endometrial biopsy and oestradiol and progesterone levels at time of endometrial and Fallopian biopsy collection.

6.3.1.1 Summary of results for section 6.3.1

Relative increase in mRNA expression over mid-secretory endometrial control			
	Proliferative (n=5)	**Mid Secretory (n=1)**	**Late Secretory (n=1)**
hBD1	12	9	21
hBD2	3	Not detected	240
hBD3	2	2	1.7
hBD4	15	5	1
SLPI	11	6	13
Elafin	3	10	43
MMP-7	2	18	19

Table 6.3.1.1 Summary of relative changes in mRNA expression of natural antimicrobials and MMP-7 across the menstrual cycle in Fallopian tube biopsies (staged using histopathology of matched endometrium and E&P levels as described in section 2.1, chapter 2).

To examine the influence of ovarian cycle stage modulation of natural antimicrobial expression in the Fallopian tube, biopsies were staged relative to the corresponding ovarian cycle stage as determined by endometrial histological stage and serum oestradiol and progesterone concentration at the time of biopsy.

Fallopian tube expression of SLPI, elafin, MMP-7 and hBD4 (figure 6.3.1.1) are influenced by ovarian cycle stage.

hBD4 mRNA levels are significantly increased in biopsy from Fallopian tube collected during the follicular phase (n=5) relative to other cycle stages ($P<0.001$) (relative to the control; mid-secretory endometrium; figure 6.3.1.1 (C)). A 4-point increase in hBD4 mRNA was observed in Fallopian tube (n=1) when compared to expression in a sample of mid-secretory endometrium.

MMP-7 mRNA levels (figure 6.3.1.1 (D)) are found to be elevated in Fallopian biopsy samples obtained from the mid and late secretory phases (according to endometrial dating and serum measurements of oestradiol and progesterone). MMP-7 mRNA is significantly lower in proliferative phase samples (n=5; $P<0.001$) relative to mid-secretory phase endometrium (control) and almost 18-point greater in the mid and late secretory phase Fallopian tube biopsies (n=1 for each).

Expression of elafin mRNA (figure 6.3.1.1 (E)) was observed to be highest in Fallopian tube collected during the late secretory phase of the menstrual cycle (n=1) (relative to mid secretory phase endometrium as control). The expression of elafin mRNA appears to be affected by the oestradiol levels (A) for these samples. There is lower mRNA expression of elafin in Fallopian tube samples collected during the proliferative phase of the menstrual cycle (n=5) expression is comparable with the decreased level of MMP-7 mRNA (6.3.1.1 (D) in the same Fallopian tube samples; this is true also for the late secretory phase biopsy.

SLPI mRNA (6.3.1.1 (F)) levels were higher (trend, no statistics) in Fallopian biopsies from the proliferative (n=5) and late secretory phase (n=1) of the menstrual cycle (relative to mid secretory phase endometrium as control), 11 and 13-point respectively. There is a slightly higher level (4 point) of SLPI mRNA expression in the Fallopian tube obtained from the mid-secretory phase.

hBD1 mRNA (6.3.1.2 (A)) levels were found to be relatively higher in Fallopian tube biopsies collected during the proliferative (n=5), mid-secretory (n=1) and late secretory phase (n=1) of the menstrual cycle, (relative to mid-secretory phase endometrium utilised as control). The greatest level of hBD1 is observed in the Fallopian tube sample during the late secretory phase (20-point).

hBD2 mRNA expression (6.3.1.2 (B)) was found to be relatively higher 230-point (over mid-secretory phase endometrial control) in the Fallopian tube sample collected from the late secretory phase. In contrast there is a less than a 2-point induction in the Fallopian tube samples collected during the proliferative (n=5) and mid-secretory phase of the menstrual cycle in comparison to the control.

Messenger RNA levels of hBD3 (6.3.1.2 (C)) are similar for the Fallopian tube biopsies obtained during the proliferative (n=5), mid-secretory (n=1) and late secretory (n=1) phases (relative to mid-secretory phase endometrium as control). hBD3 mRNA levels are unaffected with respect to the phases of the menstrual cycle that were investigated during this study.

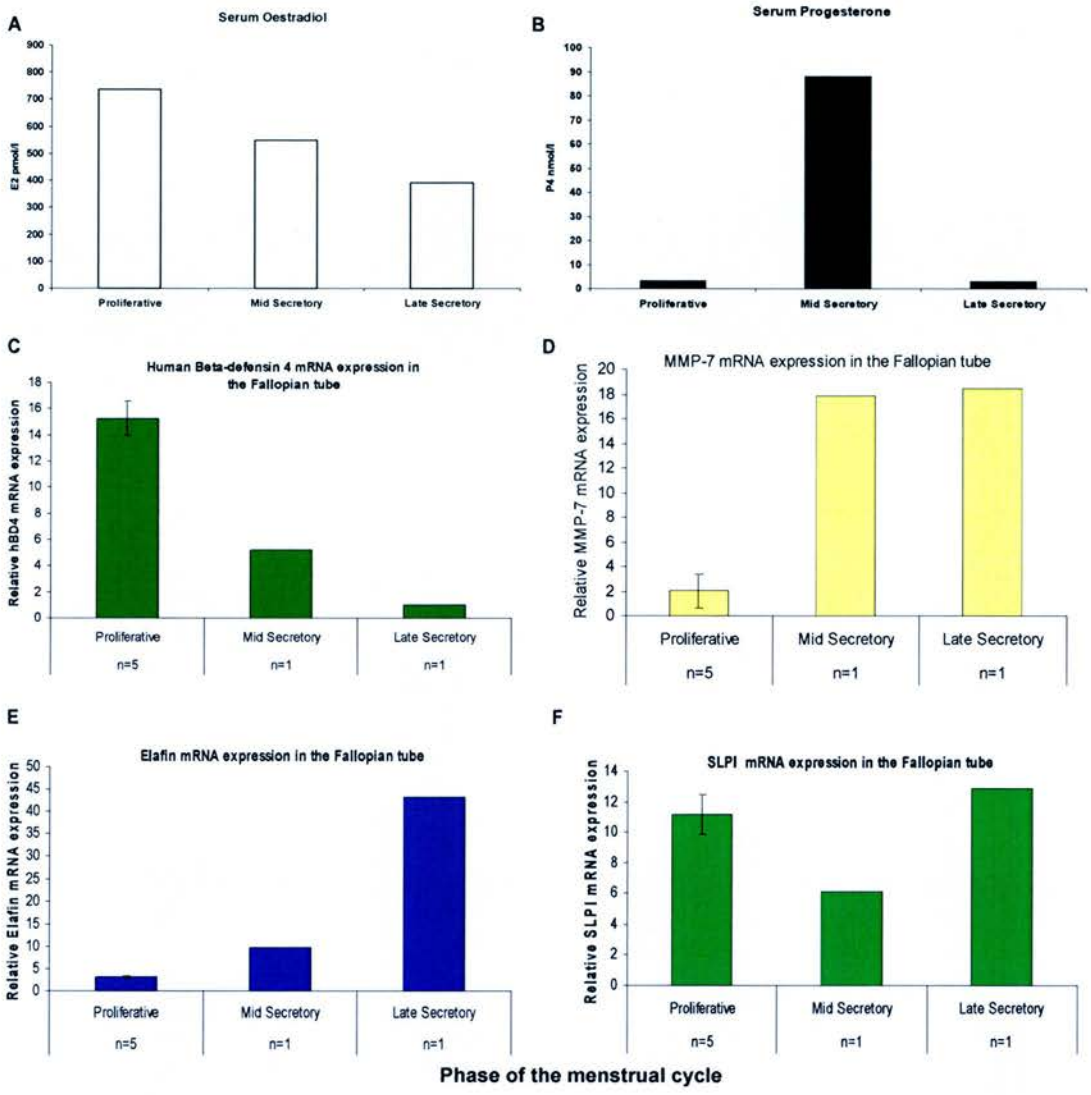


Figure 6.3.1.1 The serum levels of sex steroids at the time of Fallopian tube biopsy were measured and presented in (A) oestradiol and (B) progesterone. Graphical demonstration of the natural antimicrobials hBD4 (C), MMP-7 (D), elafin (E) and SLPI (F) mRNA expression in the Fallopian tube. Data are presented as relative changes in mRNA expression relative to a control; given a nominal value of 1, mean ± s.e.m.

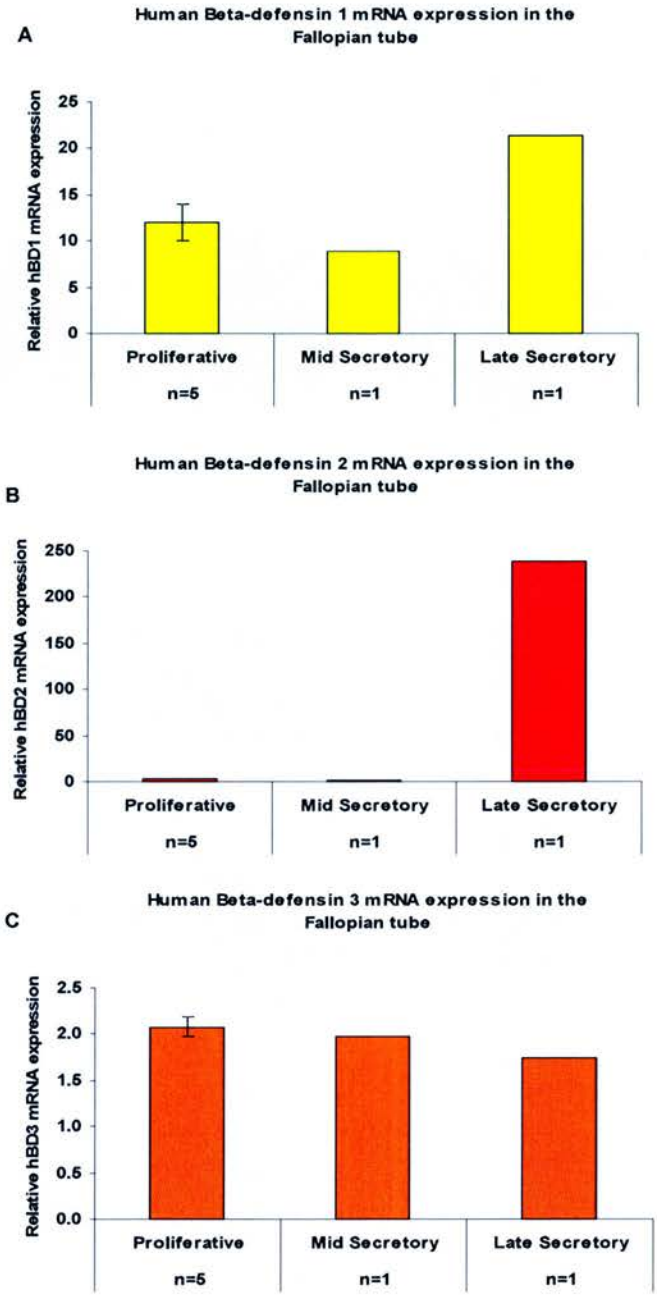


Figure 6.3.1.2 Graphical demonstration of the natural antimicrobials hBD1 (A), hBD2 (B) and hBD3 (C) mRNA expression in the Fallopian tube. Data are presented as relative changes in mRNA expression relative to a control; given a nominal value of 1, mean ± s.e.m.

6.3.2 Expression of natural antimicrobials and the matrix metalloproteinase, MMP-7 in Fallopian tube with an ectopic gestation.

6.3.2.1 Summary of results for section 6.3.2

Relative increase in mRNA expression over mid-secretory endometrial control		
	Non-ectopic gestation (n=10)	**Ectopic gestation (n=9)**
hBD1	12 +/- 3.01	11 +/- 4.48
hBD2	31 +/- 29	117 +/- 41.3 (P<0.05)
hBD3	2.4 +/- 0.31	41 +/- 17.8
hBD4	9 +/- 5.31	31 +/- 16.3
SLPI	9 +/- 1.65	19 +/- 5.7
Elafin	10 +/- 3.58	38 +/- 12.9
MMP-7	8 +/- 2.23	186 +/- 62.23

The natural antimicrobial peptides hBD2 – 4 (6.3.2.1 (A)) have an altered level of mRNA expression in the Fallopian tube samples with an ectopic gestation compared to expression in Fallopian tube without an ectopic gestation. Human β-defensin 1 mRNA (yellow) expression was unaffected by the presence of an ectopic gestation (n=9) when compared to samples without an ectopic gestation (n=10). Human β-defensin 2 (red) mRNA expression was significantly increased in Fallopian tube samples with an ectopic gestation (figure 6.3.2.1 (A), red; $P<0.05$) when compared to the non-ectopic group. The β-defensins, 3 and 4 displayed an increased tendacy in mRNA expression in the Fallopian tube samples collected from subjects with an ectopic pregnancy.

The anti-protease molecules elafin, SLPI and MMP-7 (figure 6.3.2.1 (B)) were observed to have increased mRNA expression in Fallopian tube samples collected from subjects with an ectopic pregnancy. The ratio of expression for the respective genes is altered with each showing a similar level of expression in subjects without an ectopic expression. Fallopian tube samples with an ectopic gestation demonstrated an increase in both elafin and MMP-7 mRNA expression, which was greater than that observed for SLPI mRNA. The increased expression of elafin and SLPI in the ectopic group is not statistically significant; the increase in MMP-7 is significant ($P<0.001$).

The mRNA expression profiles of elafin, SLPI and MMP-7 in individual Fallopian tube samples are represented individually in figure 6.3.2.2 (A) and (B). The expression of elafin and MMP-7 is similar, with both increased or decreased relative to SLPI in the Fallopian tube samples without an ectopic gestation (6.3.2.2 (A)). In contrast this apparent ratio is altered in the Fallopian tube biopsies from women with an ectopic pregnancy (6.3.2.2 (B)), demonstrating an increased level of MMP-7 mRNA, over that of both SLPI and elafin.

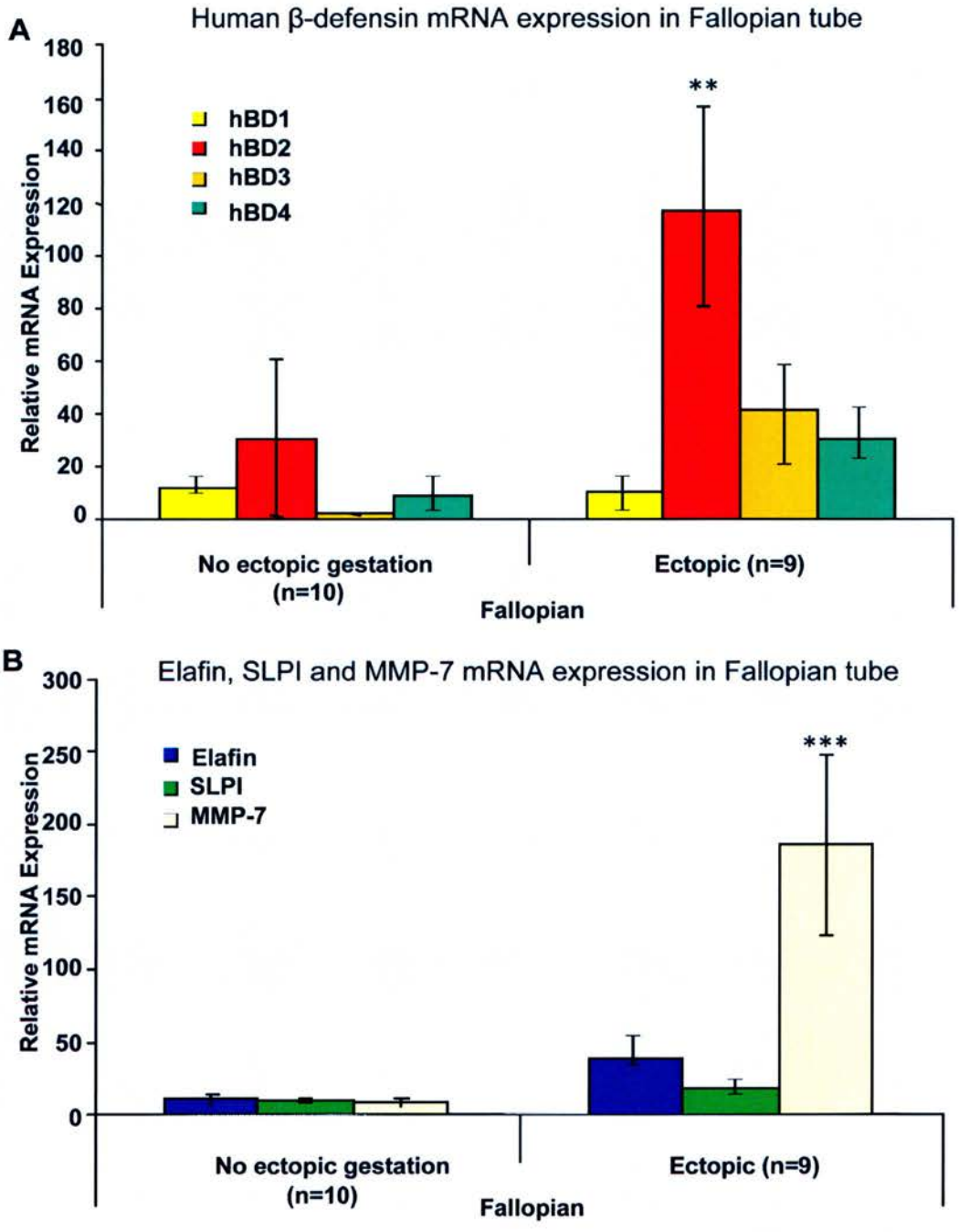


Figure 6.3.2.1 Human β-defensin 1, 2 ($P<0.05$), 3 and 4 (A) and elafin, SLPI and MMP-7 ($P<0.001$) (B) mRNA expression in Fallopian tube biopsies with (n=9) and without (n=10) an ectopic gestation. Data are presented as relative changes in mRNA expression relative to a control (mid-secretory endometrium); given a nominal value of 1, mean ± s.e.m.

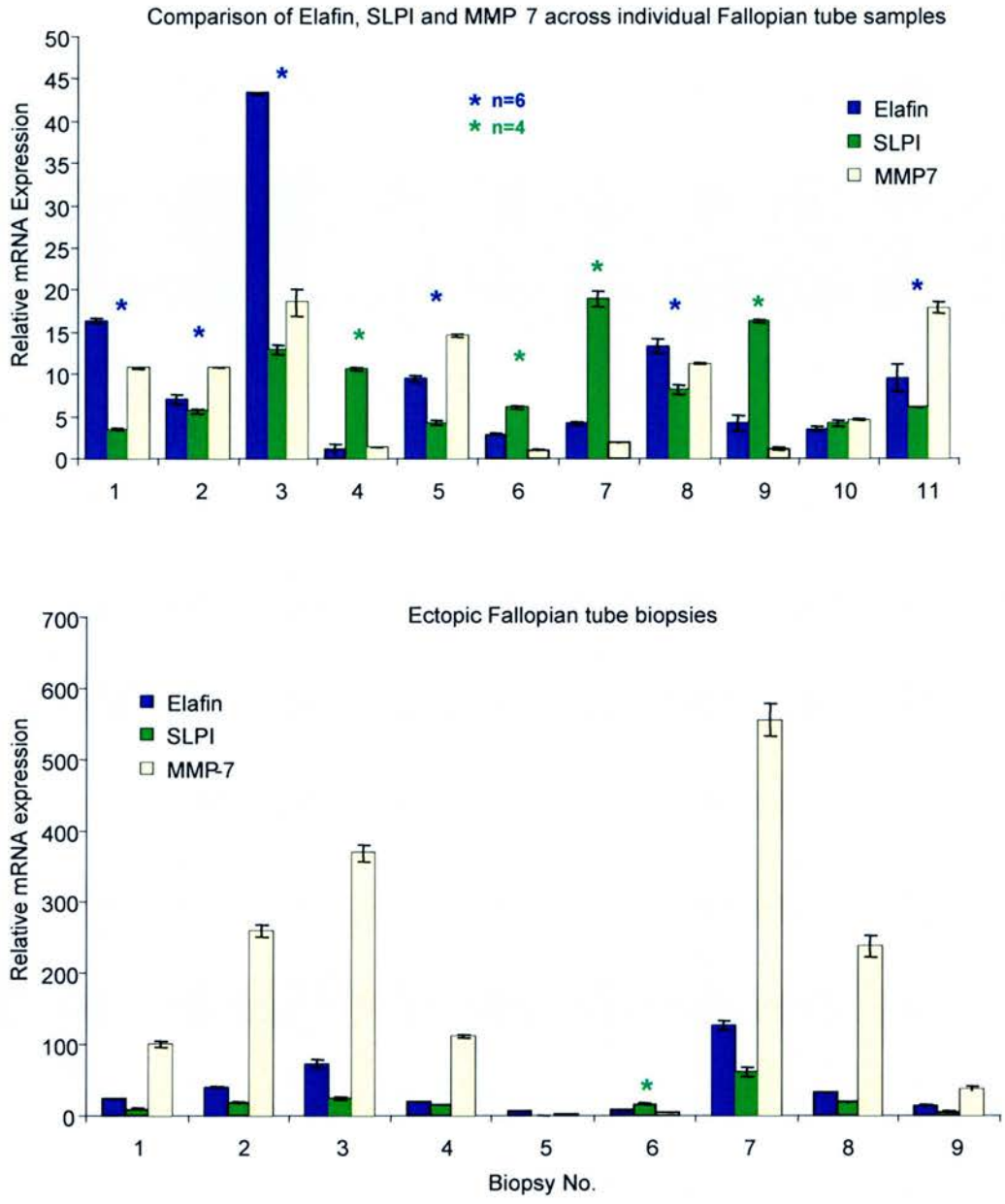


Figure 6.3.2.2 Messenger RNA expression profiles of elafin, SLPI and MMP-7 in individual Fallopian tube biopsies from subjects without (n=11; A) and with an ectopic gestation (n=9; B). Data are presented as relative changes in mRNA expression relative to a control, given a nominal value of 1, mean ± s.e.m is representative of duplicate sample wells. Note that the y axis is presented on different scales.

6.3.3 Immunohistochemical localisation of SLPI and Elafin in human Fallopian tube.

6.3.3.1 Localisation of SLPI and elafin in the Fallopian tube

Fallopian tube biopsies obtained from women undergoing surgery for benign gynaecological conditions were investigated for the presence of SLPI and elafin protein.

SLPI protein (figure 6.3.3.1) was found to be present in Fallopian tube samples obtained from women in the mid-secretory phase of the menstrual cycle (determined as described in section 2.1, chapter 2). This immunoreactivity was localised to the epithelium (B, C and D; figure 6.3.3.1) and no staining could be observed within the stromal regions. SLPI protein was found to be on the surface of the epithelial cells and in some areas gave rise to a 'blebbing' appearance (D). The level of immunoreactivity was not quantitatively determined.

Elafin protein (figure 6.3.3.2) was present in Fallopian tube biopsies collected from women in the mid and late secretory phase of the menstrual cycle (determined as described in section 2.1, chapter 2). In the mid-secretory phase biopsies (B), immunostaining was restricted to the leukocyte population (indicated by an arrow; B). There was no immunoreactivity observed in either the epithelial cells or stromal regions. In late secretory Fallopian tube samples (C and D), immunoreactivity is present in the epithelium and appears to be located in the cytoplasm.

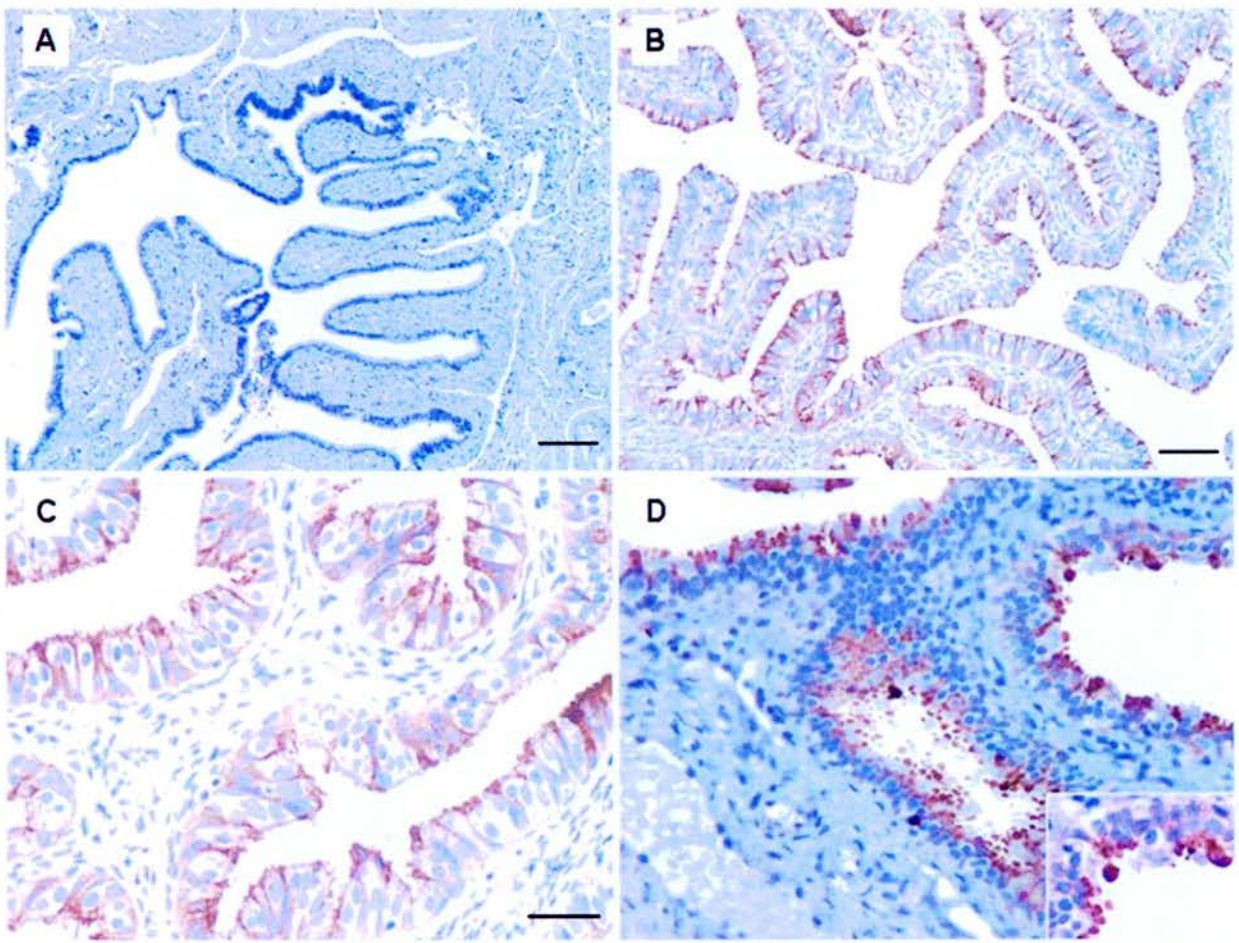


Figure 6.3.3.1 Immunohistochemical localisation of SLPI in human Fallopian tube. (A). Negative control. Primary antibody was replaced with an equimolar concentration of mouse immunoglobulin. (B). Fallopian tube biopsied during the mid secretory phase. Immunoreactivity is present in the epithelium. (C). Biopsy collected during the mid secretory phase. Immunoreactivity is present in the epithelium. (D). Immunostaining is observed in the epithelial cells. Scale bars A & B = 100 µm; C & D = 50 µm.

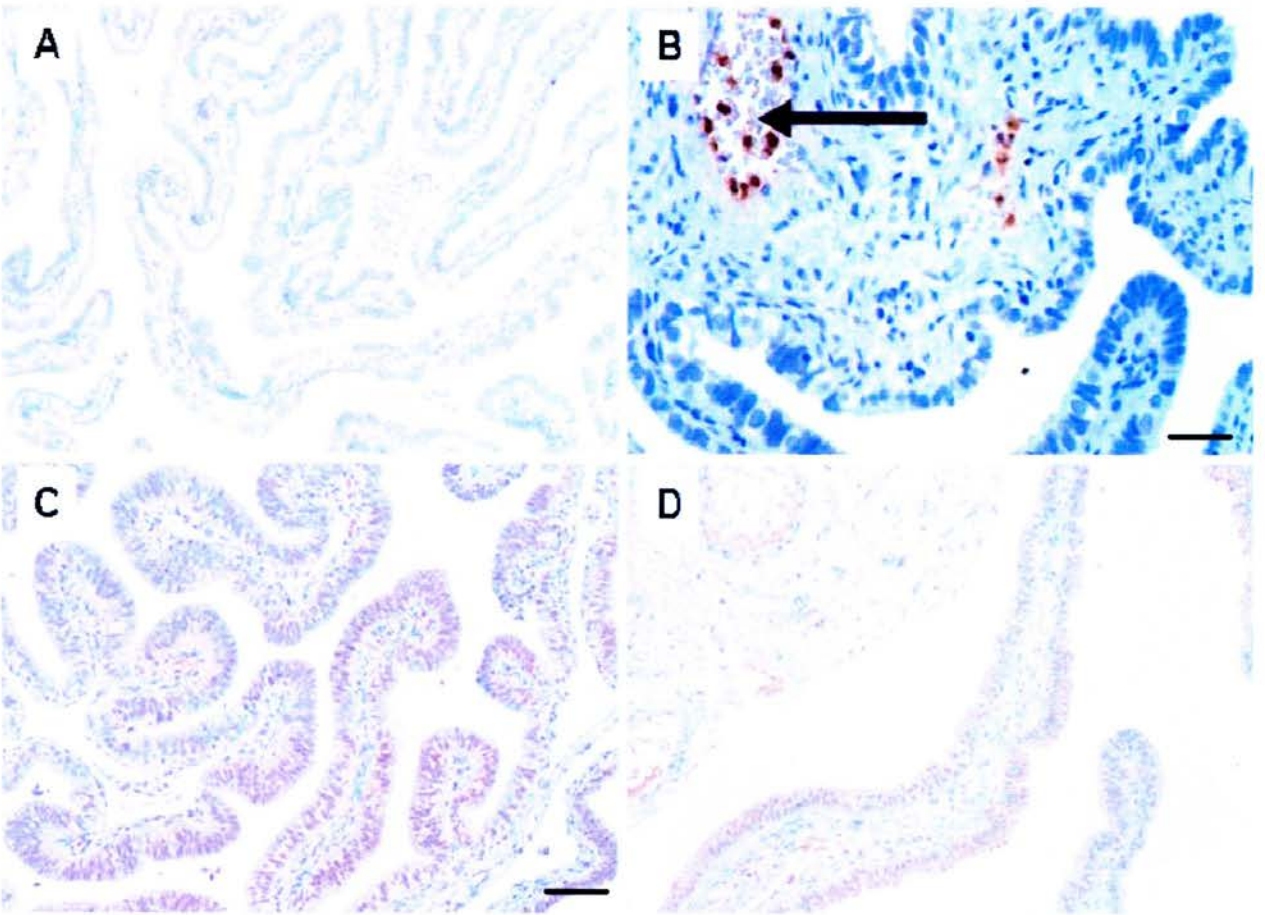


Figure 6.3.3.2 Immunohistochemical localisation of elafin in human Fallopian tube. (A). Negative control. Primary antibody was replaced with an equimolar concentration of rabbit immunoglobulin. (B). Fallopian tube biopsied during the mid secretory phase. Immunoreactivity is present in the Leukocyte population (arrow). No immunostaining was observed within the epithelial cells. (C). Biopsy collected during the late secretory phase. Immunoreactivity is present in the epithelium. (D). Immunostaining is observed in the epithelial cells in the late secretory phase. Scale bars A, C & D = 100 µm; B = 50 µm.

6.4 Discussion

6.4.1 Schematic summary of natural antimicrobial and MMP-7 mRNA expression in endometrial tissue across the menstrual cycle.

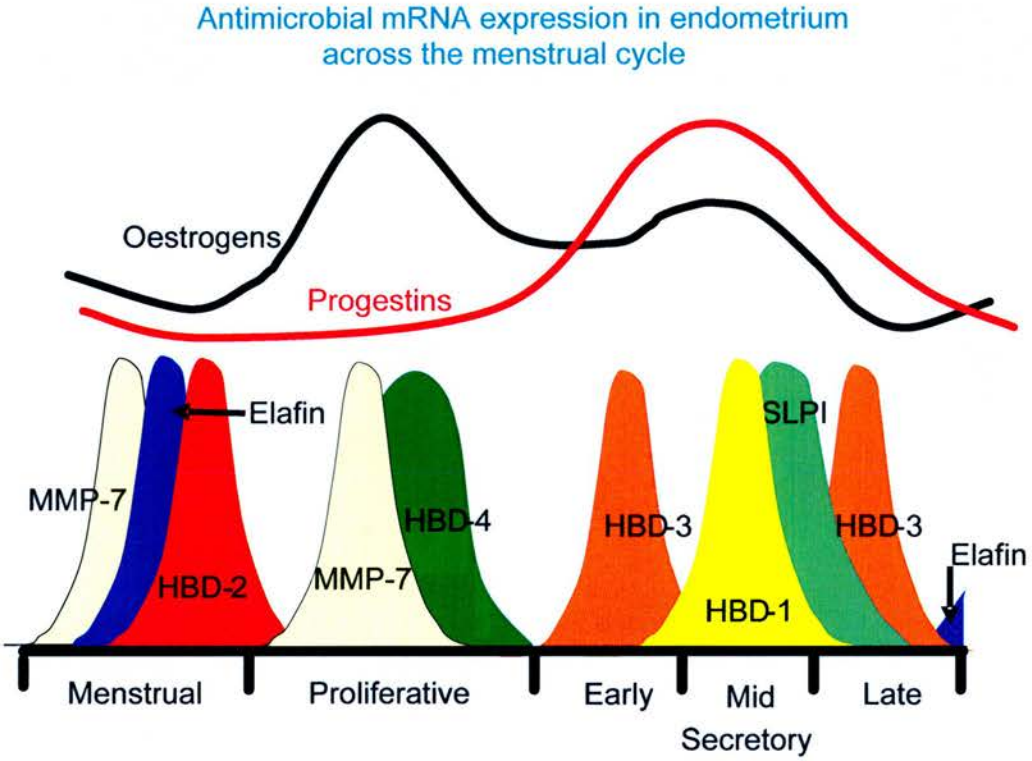


Figure 6.3.1 Schematic diagram showing the distribution of natural antimicrobial expression in endometrial tissue both in relation to the phase of the menstrual cycle and the level of circulating sex steroids, oestrogen (black) and progesterone (red). The diagram is based on reports in the literature (King, Critchley et al. 2003) and work undertaken for this thesis (chapter 5).

Based upon our knowledge of NAP expression in the endometrium and the role of the sex steroids in the regulation of natural antimicrobial expression, these preliminary data suggest that the expression of these molecules may be similarly modulated in the Fallopian tube. This is particularly evident in the expression of

elafin and hBD4, where a pattern associated with the serum levels of steroids can be observed. In the endometrium it has been previously reported that hBD4 is predominantly and maximally expressed during the proliferative phase of the menstrual cycle, when the levels of circulatory oestradiol are high. The data in this thesis demonstrate that this may also be the case in the Fallopian tube, with the greatest level of hBD4 observed in staged proliferative (follicular) samples and corresponds with the serum concentration of oestradiol measured for the same samples. It has also been shown that there is less hBD4 expression in the mid and late secretory samples corresponding to decreased levels of oestradiol measured for those samples. Further analysis of the proliferative (follicular) samples (n=5) for the level of hBD4 mRNA and the serum oestradiol for each of the individual samples (figure 6.4.1), appeared to demonstrate a direct association between expression and oestradiol levels. The expression of hBD4 could be associated with a decline or withdrawal in levels of circulatory oestradiol, in the samples investigated in this thesis, the greatest level of expression is observed in the sample with the lowest serum level of oestradiol – perhaps this sample is representative of the end of the proliferative (follicular) phase of the menstrual cycle, and it is the withdrawal of the hormones which stimulates the expression of hBD4. Further investigations using a larger sample group would enable a clearer association between the level of serum oestradiol at the time of biopsy and the expression of hBD4 mRNA. A consideration into the the possible mediators and/or mechanisms that could be attributed to the increase in this antimicrobial should be explored. It should be noted that to date none of the other antimicrobials have been found to be upregulated during the proliferative phase of the menstrual cycle for either the endometrium or the Fallopian

tube as presented herein. The reasons for this remain unclear and merits further investigation.

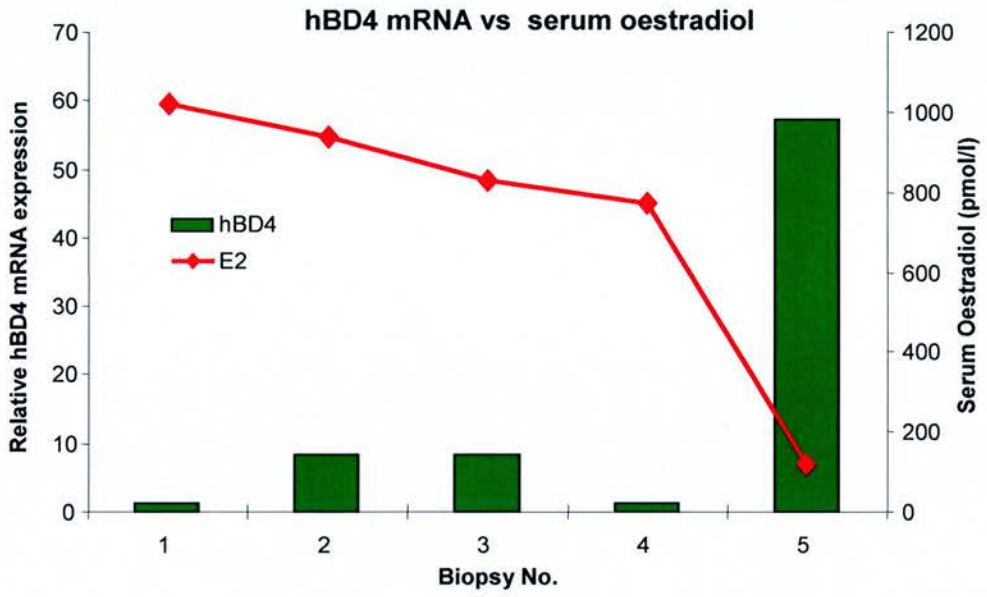


Figure 6.4.1 Comparative plot of hDB4 mRNA levels obtained for each Fallopian tube sample and compared with the serum levels of E_2 obtained at the time of biopsy.

In the endometrium it has been show previously that elafin mRNA is upregulated during the late secretory/peri-menstrual and maximally expressed during the menstrual phase of the cycle this has been proposed to be in response to progesterone withdrawal (King, Critchley et al. 2003; King, Critchley et al. 2003). In the present thesis it was demonstrated that elafin mRNA was greatly increased in the late secretory Fallopian tube biopsy. Fallopian tube biopsy samples which had been accurately staged and identified as being menstrual (as described in section 2.1, chapter 2) were unavailable at the time of writing. However, preliminary data was

obtained from pre-staged Fallopian tube biopsy samples. The level of elafin expression was consistent with that which might be expected from the menstrual phase and subsequently matched information obtained from the patients last menstrual period (LMP).

It has also been observed that there may be an inverse relationship between elafin mRNA expression and serum oestradiol levels. Increased levels of oestradiol appears to correspond with decreased levels of elafin mRNA in the samples that we investigated (n=7). The expression of elafin mRNA is highest in the Fallopian tube collected during the late secretory (luteal) phase and with lower levels of serum progesterone. There is a low serum progesterone level in the samples obtained from the proliferative phase; however, there is a high oestradiol level. Thus, it may be possible that oestradiol has an inhibitory effect upon the expression of elafin or mediators/mechanisms required for the expression of elafin.

SLPI has been shown to be upregulated by progesterone *in vitro* in the breast cell line and within this thesis the endometrial epithelial cell line Hec-1A. In the endometrium SLPI has been reported previously and confirmed within this thesis (chapter 5) to be maximally expressed during the mid-secretory phase of the menstrual cycle, when circulatory progesterone levels are high. However, in the Fallopian tube biopsies investigated there was an increased level of SLPI in those collected during the proliferative (follicular) phase and late secretory (luteal) phase and was greater than the level of SLPI observed in the mid-secretory phase (luteal) biopsy. However, the numbers are too small to allow for any conclusive statements.

It has also been observed that the expression of SLPI in the Fallopian tube samples studied is opposite to the level of serum progesterone. There may be a difference between the Fallopian tube and the endometrium in the expected level of SLPI expression. An alternative is that it may suggest that the local levels of sex steroids within the Fallopian tube may not be representative of that detected in the peripheral blood samples. There is no precedence that endometrial cycle stage is necessarily in synchrony with the Fallopian tube or indeed that the Fallopian tube responds in relation to the stages of the ovarian cycle.

In order to further discover the proposed role of MMP-7 in the expression of elafin and or SLPI, this too was investigated in the menstrual staged Fallopian tube samples in order to elucidate any similarities in the patterns of expression. MMP-7 has been previously described to be maximally expressed during the menstrual and proliferative phase in the endometrium (Rodgers, Osteen et al. 1993; Rodgers, Matrisian et al. 1994) and was demonstrated within this thesis (chapter 5). However, the analysis of MMP-7 mRNA expression demonstrates maximal expression in the mid and late secretory (luteal) phase Fallopian tube biopsies, with relative reduced levels in those collected during the follicular (proliferative) phase. It could be proposed that if there is a direct or indirect relationship between the expressions of MMP-7 with elafin and/or SLPI there may be a 'lag'. In the endometrium MMP-7 expression is shown to be increased in the menstrual phase along with elafin, however, increased MMP-7 expression has also been observed in the proliferative phase, whilst elafin is not expressed. In the Fallopian tube it appears that the maximal expression of both MMP-7 and elafin is coupled in the biopsies collected in

the late secretory (luteal) phase. Whereas a smaller increase in the levels of elafin mRNA are observed in the mid secretory (luteal) phase along with high levels of MMP-7. In contrast both demonstrate low expression levels in the proliferative phase Fallopian tube biopsies. It may also be interesting to note that this coincides with an increased level of SLPI in the proliferative (follicular) phase samples.

Analysis of the mRNA levels of the β-defensins in the stage determined Fallopian tube biopsies have also demonstrated differential expression. Human β-defensin 1 is shown to be expressed at a similar level in the proliferative (follicular) and mid secretory (luteal) phase biopsies, whilst the late secretory (luteal) phase sample demonstrates almost a doubled level of hBD1 mRNA. Previously, it has been shown that hBD1 is constitutively expressed through all phases of the cycle in endometrium (Fleming, King et al. 2003; King, Critchley et al. 2003); it has also been proposed to have a constant level of expression in other mucosal regions (Pazgier, Hoover et al. 2006). The low group numbers currently presented prevent conclusive analysis. However, the expression of hBD1 in the Fallopian tube in relation to the menstrual cycle has not previously been described. In contrast hBD2 was found to be highly expressed only in the late secretory (luteal) sample, low levels of hBD2 mRNA are observed in the proliferative (follicular) phase, but, was undetected in the mid secretory (luteal) biopsy. Human β-defensin 2 has been described as being maximally expressed during the menstrual phase in endometrium (Fleming, King et al. 2003; King, Critchley et al. 2003), it has further been demonstrated that hBD2 expression is inhibited in the presence of progesterone (Fleming, King et al. 2003), (chapter 3). The serum level of progesterone for the late secretory (luteal) sample is

low which may also support these observations for the Fallopian tube. Human β-defensin 3 mRNA is observed at a similar but relatively low level for all Fallopian tube biopsy samples, hBD3 in the endometrium was reported to be greatest in the late secretory phase and this is thought to be in response to an increase in cytokines and the influx of IFNγ secreting neutrophils (King, Fleming et al. 2003).

It is likely that the expression of natural antimicrobials in the Fallopian tube is differential according to the stage of the ovarian cycle at the time of collection. Fallopian tube biopsies were collected across the ovarian cycle from women undergoing surgery for benign gynaecological procedures and were compared with the Fallopian tube samples collected from women with an ectopic pregnancy. The mRNA expression levels of the human β-defensins are altered between the two groups with the exception of hBD1 which is unchanged. Human β-defensin 2 mRNA is significantly ($P<0.05$) greater in the Fallopian tube of woman with an ectopic pregnancy, which may indicate the potential for this defensin to be further investigated as a diagnostic marker for this condition. It may also offer further opportunities from our understanding of the conditions required for hBD2 expression and allow for the elucidation of other candidates and/or mechanisms. However, hBD2 is also an inflammatory mediator and it is not possible with the current study to differentiate between a causal or effective role in ectopic pregnancy. Human β-defensins 3 & 4 are also increased in the Fallopian tube of women with an ectopic pregnancy, further indicating a role for these molecules in an ectopic gestation and further study is merited.

The investigation into the expression of elafin, SLPI and MMP-7 in Fallopian tube were also undertaken. An increase in mRNA levels was seen for all three molecules in the group of subjects with ectopic pregnancy, with MMP-7 being significant ($P<0.001$). SLPI mRNA was only slightly elevated, whilst elafin was 30-point greater in Fallopian tube with an ectopic gestation. The MMPs have been identified as having a number of roles in innate immunity including the processing of cytokines (Gearing, Beckett et al. 1995; Opdenakker, Van den Steen et al. 2001), and defensins into their active forms (Ayabe, Satchell et al. 2002; Shirafuji, Tanabe et al. 2003; Weeks, Tanabe et al. 2006). They are also involved in the chemotaxis and movement of leukocytes through regions of inflammation (Murphy and Gavrilovic 1999; Vaday, Hershkoviz et al. 2000; Vaday, Franitza et al. 2001). Thus, it is possible that MMP-7 has a role in relation to the expression of natural antimicrobials or in the modulation of an inflammatory response towards an ectopic gestation. A role has been suggested for the MMPs in the post infection scarring and pathology associated with *Chlamydia.* The TNFα mediated upregulation and subsequent monocyte infiltration increases the level of damage to the extracellular matrix (ECM) and deposition of collagen (Ault, Kelly et al. 2002). The involvement of elafin (van Bergen, Andriessen et al. 1996) and SLPI (Angelov, Moutsopoulos et al. 2004) in wound repair may explain any association between these molecules in the Fallopian tube with an ectopic gestation.

The analysis of the samples within the Fallopian tube both with and without an ectopic gestation revealed interesting differences in the ratio of elafin, SLPI and MMP-7 mRNA expression. In the control group (no ectopic), the expression of

elafin and MMP-7 appear to be increased together over SLPI (*n=6), or both decreased in relation to SLPI (*n=4). Further, to this observation it can also be seen that these two expression ratio/relationships are represented almost 60:40 – in that out of 11 samples, 6 show increased elafin and MMP-7, and 4 show the converse decreased elafin and MMP7, increased SLPI. One sample (10) demonstrates similar levels for each of the genes. Fallopian samples with an ectopic pregnancy did as previously described show an increased level of mRNA for elafin, SLPI and MMP-7 in comparison with the Fallopian tube samples with no ectopic gestation. However, the ratios are altered; the expression level of MMP-7 mRNA is the most increased in the majority of the samples. As with the Fallopian tube samples without an ectopic gestation, there is also an increased level of elafin along with MMP-7. The least amount of change is observed with the level of SLPI mRNA and in contrast to the samples without an ectopic gestation, only exceeds the level of elafin and SLPI expression in one of the nine samples (figure 6.3.2.2 B [*]). This may be a significant observation for the further understanding of the changes involved in tubular ectopic pregnancy. However, whether this change is causal or consequential would also need to be elucidated.

SLPI and elafin protein were detected in the Fallopian tube obtained from women undergoing surgery for benign gynaecological conditions. Immunoreactivity for SLPI was present in the epithelium of Fallopian tube sampled during the mid secretory (luteal) phase. This was in agreement with the observation of increased SLPI mRNA in mid-secretory (luteal) phase also. However, greater amounts of SLPI mRNA were observed in the proliferative (follicular) and late secretory (luteal)

phase samples (figure 6.3.1.1.F). A larger number of samples are required to be investigated before any conclusive discussions are possible. Elafin immunoreactivity was found to be localised to the leukocyte population in mid secretory (luteal) phase biopsies. The epithelial cells of samples obtained from the late secretory (luteal) phase were found to have staining for elafin. The highest level of elafin mRNA was also found to be in the samples obtained from the late secretory (luteal) phase (figure 6.3.1.1 E). However, the amount of immunoreactivity was not quantitatively measured and too few samples were examined due to time restrictions.

In summary, these preliminary data suggest that the expression of natural antimicrobials in the Fallopian tube is subjected to changes governed by the ovarian cycle. This is similar to the observations of the differential expression of natural antimicrobials in the endometrium across the menstrual cycle (King, Critchley et al. 2003). However, it seems likely that the expression of natural antimicrobials in the Fallopian tube is asynchronous to that observed in the endometrium. The expression of hBD4 in the Fallopian tube appears to be asscociated with circulating levels of oestradiol. This may be significant when considered with the previous findings of maximal expression in endometrium collected from the proliferative phase of the menstrual cycle (King, Fleming et al. 2003).

The presence of an ectopic pregancy increased the level of most natural antimicrobials and MMP-7 when compared to Fallopian tubes without an ectopic gestation. The expression levels of SLPI, elafin and MMP-7 mRNA have been shown to have an association with each other. The expression of SLPI was found to be greater than both elafin and MMP-7 (n=4) or lower than both elafin and MMP-7 (n=6), (figure 6.4.2 A). Furthermore, this pattern of expression appears to be altered in the presence of an ectopic gestation (figure 6.4.2 B). The expression of MMP-7 mRNA was greatly increased; similarly elafin was increased but remained below that of MMP-7. Perhaps it is not the expression of these molecules but their ratio in relation to one another that should be considered. Further analysis of the expression pattern and possible association between SLPI, elafin and MMP-7 was also undertaken with decidual groups (chapter 7), and via the treatment of Hec-1A cells with SLPI and MMP-7 in chapter 8 of the current thesis.

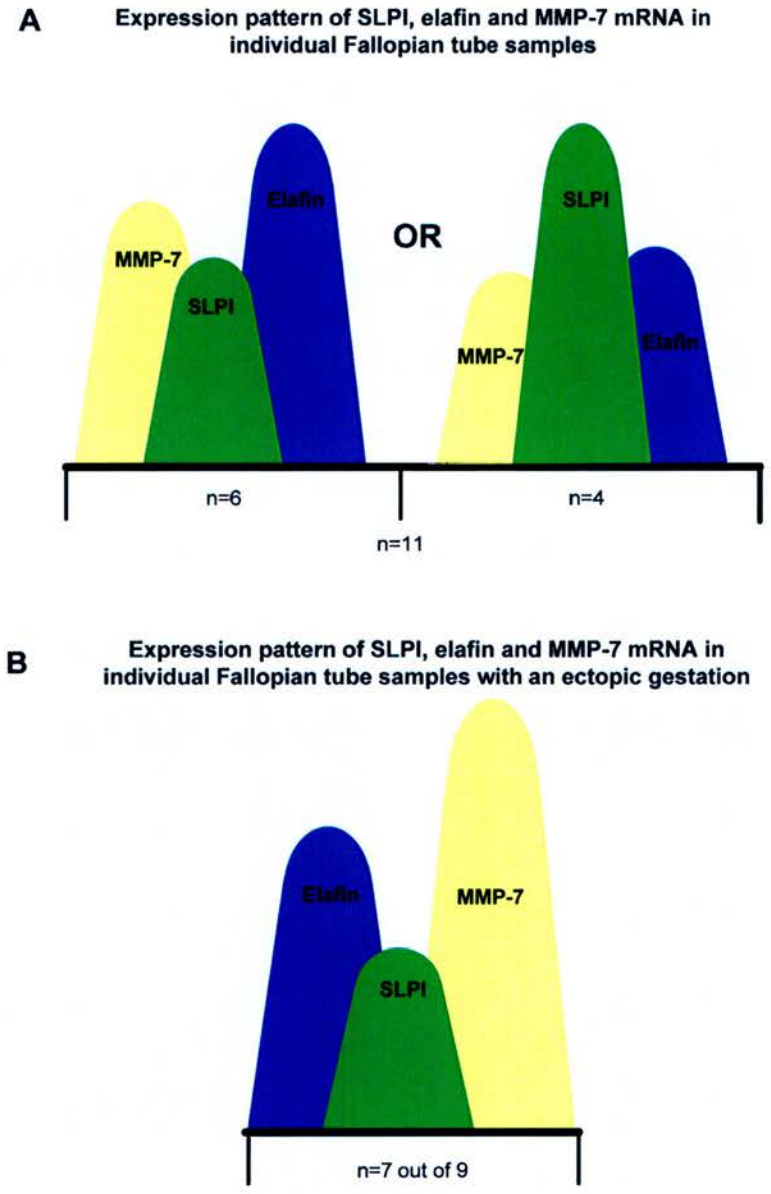


Figure 6.4.2 Schematic summary derived from the data presented in figure 6.2.5.2. In Fallopian tube without an ectopic gestation (A), SLPI was found to be lower than elafin and MMP-7 (n=6) or higher than elafin and MMP-7 (n=4). It was also apparent that elafin was greater than MMP-7 (n=7). In Fallopian tube with an ectopic gestation (B), the pattern(s) were altered. MMP-7 was found to be expressed at a higher level elafin. Both MMP-7 and elafin were found to be greater than SLPI in 8 out of the 9 samples investigated.

Chapter 7:

Natural antimicrobial expression in first trimester decidua from women with ectopic gestation and miscarriage.

Chapter 7: Natural antimicrobial expression in first trimester decidua from women with ectopic gestation and miscarriage.

7.1 Introduction

Successful human reproduction involves the implantation of a fertilised oocyte into endometrial tissue, where sufficient nutrient support is available to allow for development and maturity (Marx, Arck et al. 1999). In preparation for implantation the human endometrium undergoes the process of decidualisation, involving both functional and morphologically distinct changes in the tissue (Loke, King et al. 1995; King 2000). In the event of an ectopic pregnancy, implantation occurs outwith the endometrium. The epithelium of the Fallopian tube can not facilitate a successful pregnancy and does not undergo decidualisation (Stewart-Akers, Krasnow et al. 1997). However, the uterine endometrium still undergoes the process of decidualisation (Stewart-Akers, Krasnow et al. 1997). Thus, there have been studies into the comparative differences between the decidua of a normal intra-uterine pregnancy with that of an empty uterus arising from an ectopic gestation (Marx, Arck et al. 1999).

Natural antimicrobials have been found to be associated with a number of mucosal surfaces and serve to function as key members of the innate immune response. Natural antimicrobials have been shown to be expressed throughout the female reproductive tract (summarised in figure 1.6.1) which is suggestive of a pivotal role for these molecules in immune regulation. In chapter 6 it was shown that the presence of an ectopic gestation gave rise to an increase in the expression of natural antimicrobial mRNA in the Fallopian tube. This was suggestive of a role for these

molecules in the inflammatory response associated with an ectopic pregnancy. The expression of natural antimicrobials during early pregnancy has been previously described. SLPI has been shown to be more highly expressed than in cyclic endometrium (maximal during the mid-secretory phase), (within this thesis; chapter 5) (King, Critchley et al. 2000); hBD1, hBD2 and granulysin have also been shown to be expressed (Fleming, unpublished), (King, Critchley et al. 2003) and elafin, within this thesis, chapter 5.

The expressions of natural antimicrobials in the decidua of pathologic situations such as the failure in pregnancy resulting from an ectopic gestation or in miscarriage have not been described previously.

7.2 Materials and Methods

7.2.1 Sample collection

First trimester decidua was obtained from patients undergoing surgical termination of pregnancy (STOP; n=6); surgical management of miscarriage (n=5) and ectopic gestation (n=10) and detailed in table 7.2.1.1. All samples were examined histologically (H&E) to exclude the presence of trophoblast.

Tissue Sample	Age	Group	Parity	Gestation	Procedure
1	35	1	1+2	10+6	Evacuation of Retained Products of Conception
2	31	1	1+0	10+6	Evacuation of Retained Products of Conception
3	34	1	0+0	12+6	Evacuation of Retained Products of Conception
4	30	1	0+0	14+1	Evacuation of Retained Products of Conception
5	28	1	1+1	11+1	Evacuation of Retained Products of Conception
6	28	1	0+0	9+6	Evacuation of Retained Products of Conception
1	33	2	3+3	10+5	Surgical Termination of Pregnancy
2	28	2	0+0	10	Surgical Termination of Pregnancy
3	22	2	1+1	8+6	Surgical Termination of Pregnancy
4	24	2	0+0	10	Surgical Termination of Pregnancy
5	29	2	1+3	10+6	Surgical Termination of Pregnancy
1	30	3	0+0	6+2	Diagnostic Laparoscopy
2	25	3	1+1	7+1	Diagnostic Laparoscopy
3	40	3	1+0	8+3	Diagnostic Laparoscopy
4	31	3	2+1	5+4	Diagnostic Laparoscopy
5	28	3	2+2	5+3	Diagnostic Laparoscopy
6	26	3	0+0	6+0	Diagnostic Laparoscopy
7	26	3	1+0	6+6	Diagnostic Laparoscopy
9	33	3	0+0	11+1	Diagnostic Laparoscopy
10	35	3	0+1	6+1	Diagnostic Laparoscopy

Table 7.2.1.1 Uterine decidua collected from women (age 22-40) undergoing surgical management of miscarriage (n=6; Group 1), surgical termination of pregnancy (n=5; Group 2) and the surgical management of ectopic pregnancy (n=10; Group 3).

7.2.2 RNA extraction and Q-RT-PCR

RNA was extracted from frozen biopsy samples stored in RNA later, using the Qiagen proteinase K protocol and cDNA prepared, as detailed in chapter 2. Expression levels of SLPI, Elafin, MMP-7 and hBD1-4 mRNA were determined using quantitative real time PCR (as detailed in section 2.3.1).

7.2.3 Immunohistochemistry

Elafin and SLPI were localised in first trimester decidua (STOP, n=6; Miscarriage, n=5; Ectopic, n=10), using the protocol detailed in chapter 2 (section 2.6).

7.2.4 Statistical Analysis

The PCR results in this chapter did not conform to normal distribution and were subject to non-parametric statistical analysis. Fisher's protected least significant difference (PLSD) was used to assign individual differences (PRISM).

7.3 Results

7.3.1 Natural antimicrobial expression in human first trimester decidua

Decidua was obtained from women undergoing surgical management of ectopic pregnancy (n=10), surgical management of miscarriage (n=5) and termination of pregnancy during the first trimester (STOP; n=6).

Elafin mRNA (figure 7.3.1.1 (A)) expression levels were increased in the uterine decidua from subjects with an ectopic gestation, when compared with expression levels in decidua where the gestation was uterine (not statistically significant). Messenger RNA levels for elafin are also raised in the decidual samples obtained from woman with miscarriage (n=5).

SLPI mRNA (figure 7.3.1.1 (B)) levels are increased in the decidua from subjects with an ectopic gestation (not statistically significant) when compared to expression in decidua of STOP or miscarriage. There was no difference in the mRNA expression levels of SLPI in decidua from women with miscarriage or having a STOP.

Messenger RNA for MMP-7 was significantly increased in decidua from women with an ectopic pregnancy when compared to the expression in decidua from women with either a miscarriage or STOP (figure 7.3.1.1 (C); $P<0.01$ (n=10)). There was no difference in the level of MMP-7 mRNA in decidua between the STOP and miscarriage subjects.

In order to view the level of changes in mRNA for elafin, SLPI and MMP-7 in relation to one another the data are plotted on the same scale figure 7.3.1.1 (D).

The mRNA expression profiles of elafin, SLPI and MMP-7 in individual decidual samples are represented individually in figure 7.3.1.2 (A) ectopic gestation, (B) STOP and (C) miscarriage. In the decidua from women with an ectopic gestation (A), MMP-7 mRNA levels were increased substantially over the level of both SLPI and elafin mRNA. Elafin mRNA was found to be increased over the levels of SLPI. SLPI was found to be the highest expressed in the decidua obtained from women with a STOP (B), with lower levels of elafin and MMP-7 mRNA observed. In decidua from failed intrauterine pregnancy (C), SLPI mRNA was reduced and the levels of elafin and MMP-7 increased. The patterns of expression of SLPI, elafin and MMP-7 are altered between the decidual groups

Human β-defensins 1-4 mRNA (figure 7.3.1.3 (A)) levels are found to be altered in the decidua from women with an ectopic pregnancy. The mRNA expression profiles for all four of the defensins are presented on the same scale (A), and on separate scales respective of their level of expression (B – E). Human β-defensin 1 (B) mRNA levels are decreased in the decidua from women with an ectopic pregnancy when compared with expression in decidua from women with miscarriage or STOP (non-significant). In contrast the mRNA levels of hBD2, hBD3 ($P<0.05$ decidua from ectopic gestation vs. STOP) and hBD4 all demonstrate an increase in expression in decidua with an ectopic gestation. Human β-defensins 2 and 4 also

exhibit an increase in decidua from subjects with a failed intrauterine pregnancy (miscarriage).

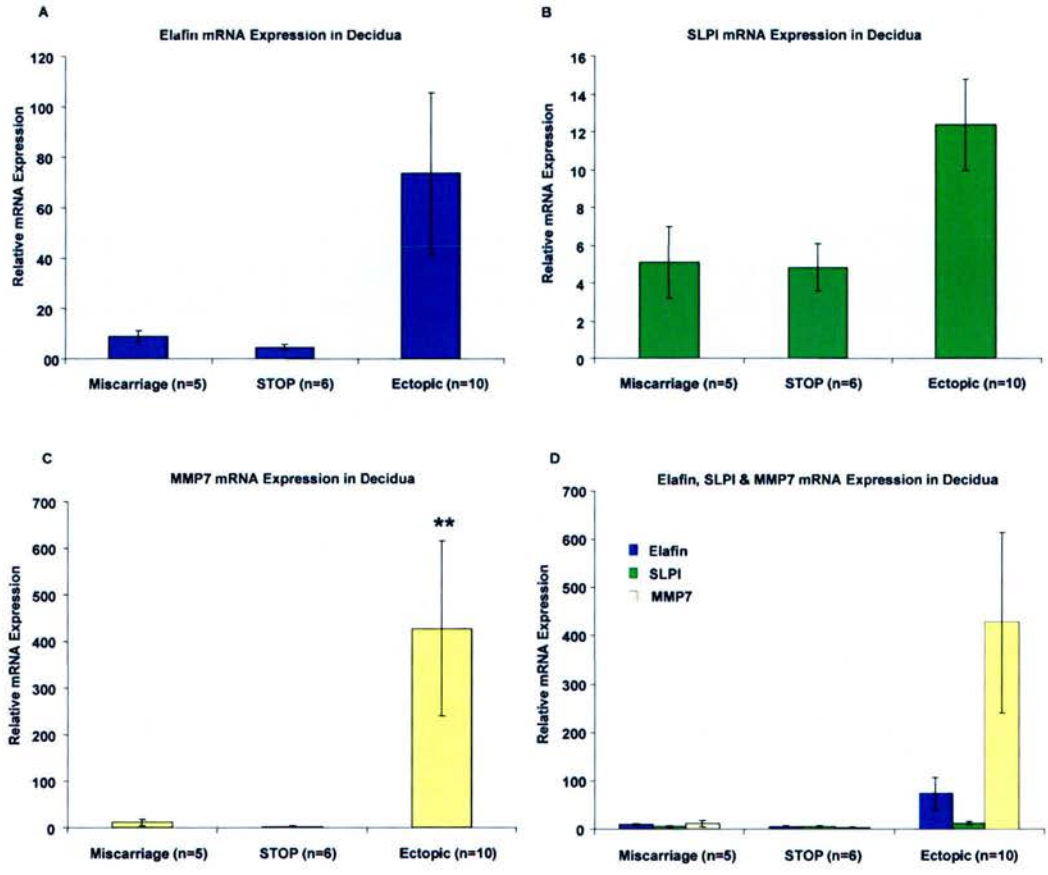


Figure 7.3.1.1 Elafin (A; blue), SLPI (B; green), MMP-7 (C; yellow; $P<0.01$ decidua from ectopic pregnancy vs. STOP) and all on the same scale (D). Data are presented as point changes in mRNA expression relative to a control (comparator obtained from a sample of early secretory endometrium), given a nominal value of 1, mean ± s.e.m.

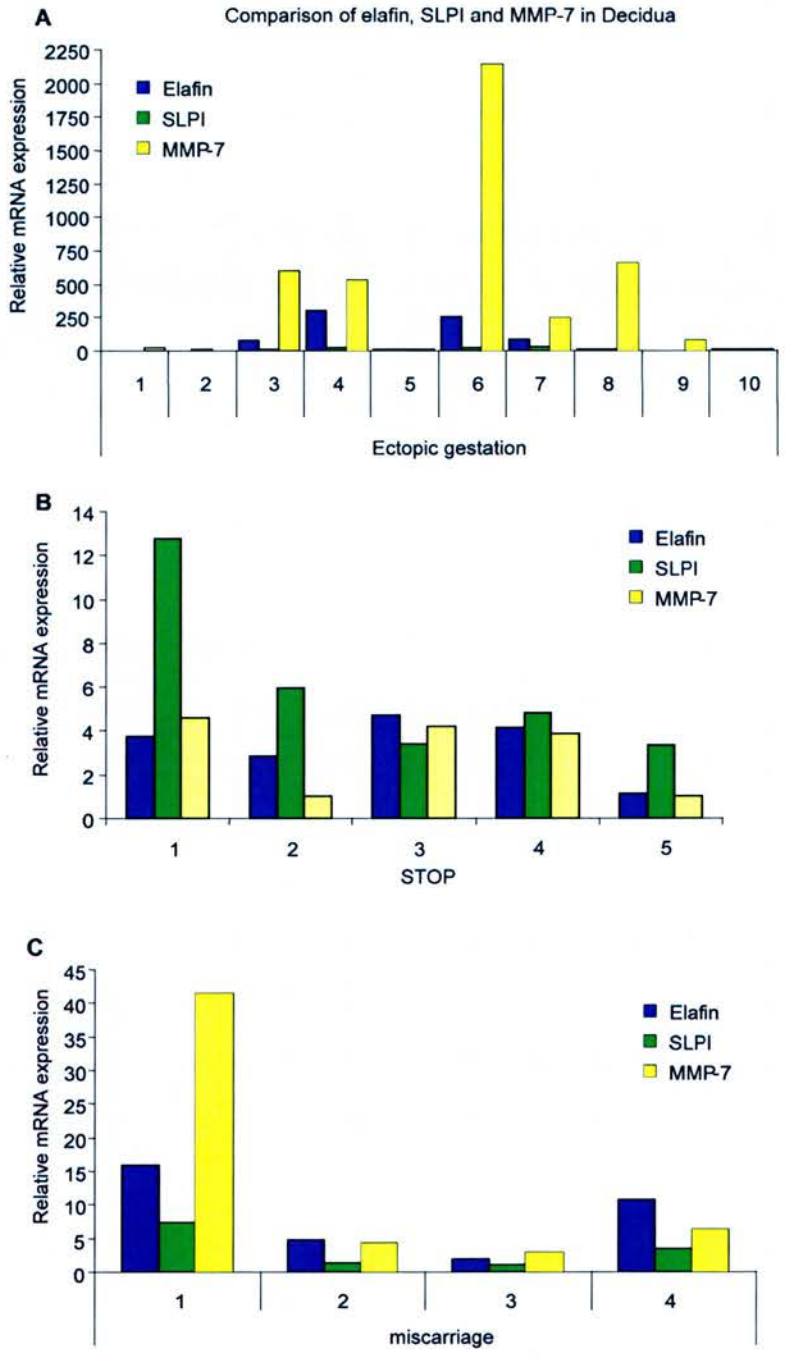


Figure 7.3.1.2 Expression profiles of elafin, SLPI and MMP-7 in decidua from ectopic gestation (A; n=10); STOP (B; n=5) and miscarriage (C; n=4). Data are presented as relative changes in mRNA expression relative to a comparator, given a nominal value of 1.

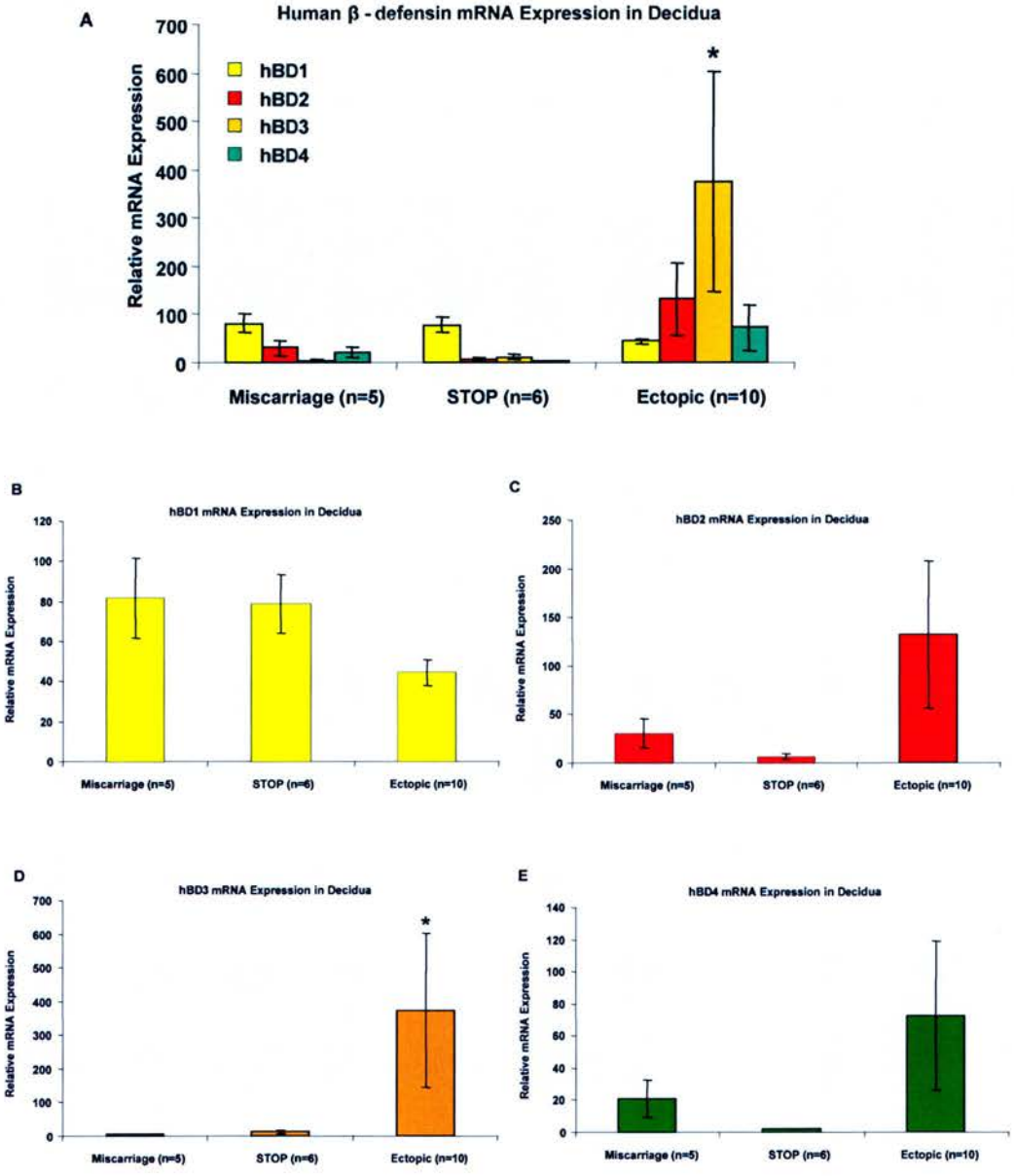


Figure 7.3.1.3 hBD1 (B; yellow), hBD2 (C; red), hBD3 (D; orange; P<0.05 ectopic vs. stop) and all on the same scale (A). Data are presented as relative changes in mRNA expression relative to a control (comparator obtained from a sample of early secretory endometrium), given a nominal value of 1, mean ± s.e.m.

7.3.2 Immunohistochemical localisation of elafin and SLPI in first trimester decidua.

Elafin protein was found to be present in the decidua obtained from women with an ectopic gestation (D) and in women with miscarriage (C), (figure 7.3.2.1). This immunoreactivity was observed in the epithelium with some diffuse staining in the stroma. In STOP decidua (B); there was no immunoreactivity observed in the stroma or epithelial cells. Some immunostaining was observed within the leukocyte population within the blood vessels (*). There appeared to be more elafin present in the decidua of miscarriage patients (C) and decidua from women with an ectopic pregnancy (D) when compared to decidua from women undergoing surgical termination of pregnancy (STOP). However, this was not quantitatively measured.

SLPI protein (figure 7.3.2.2) was present in all types of decidua, from women with STOP (B), miscarriage (C) and with an ectopic gestation (D). In STOP (B) decidua immunoreactivity was observed to be intense within the epithelium with some diffuse immunostaining observed within the stroma. In decidua obtained from miscarriage patients (C) there was substantial immunoreactivity in the epithelium and throughout the stroma, however, the cytoplasm and nucleus of stromal cells did not exhibit any immunostaining. In decidua obtained from women with an ectopic pregnancy, there was intense SLPI protein immunoreactivity observed in the glandular epithelium. The amount of SLPI protein appeared to be increased in the decidua from an ectopic gestation. However, this was not quantitatively determined.

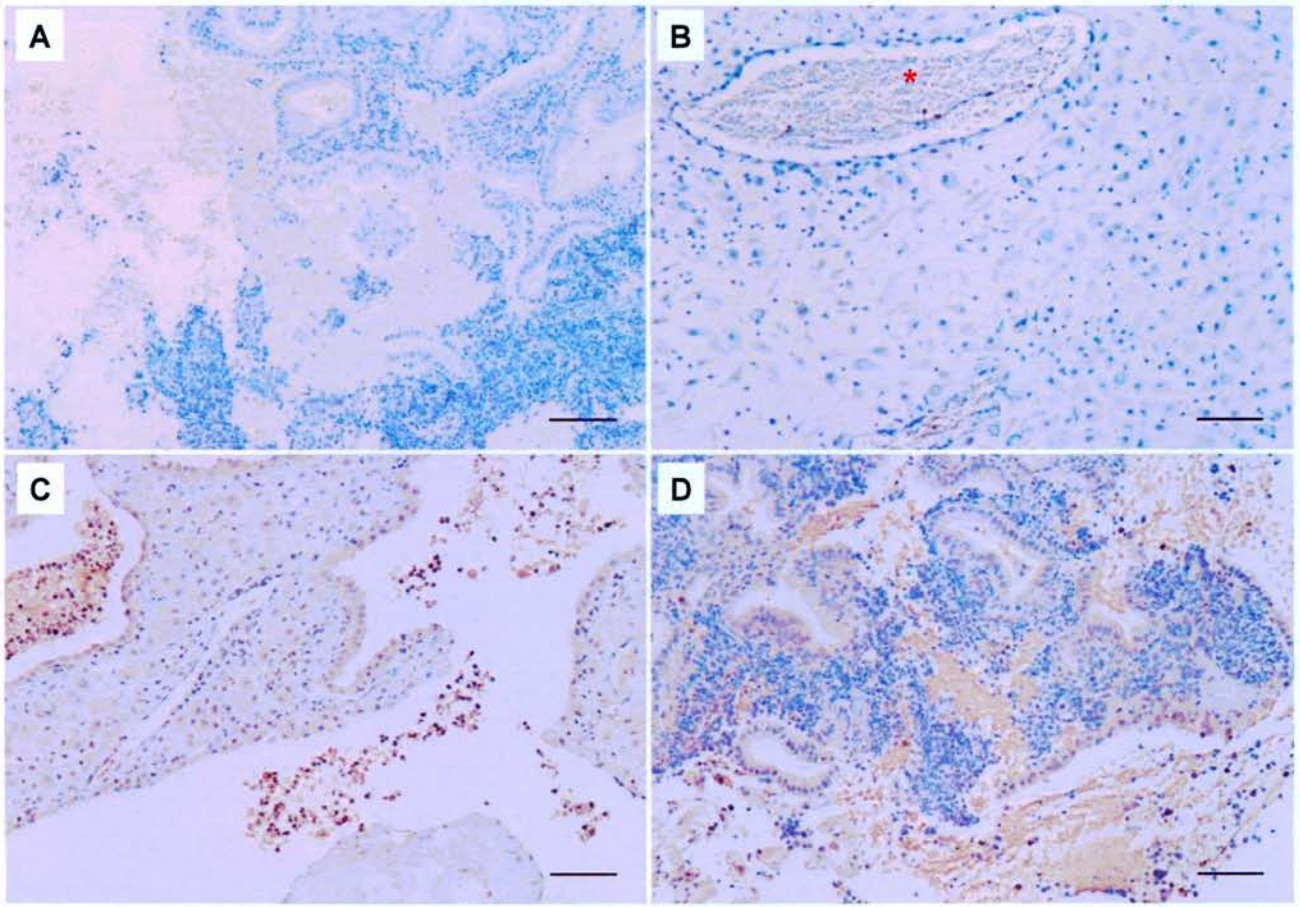


Figure 7.3.2.1 Immunohistochemical localisation of elafin in human decidua obtained from STOP (B), miscarriage (C) and ectopic gestation (A&D). (A). Negative control (decidua from ectopic gestation). Primary antibody replaced with an equimolar concentration of rabbit immunoglobulin. (B) Little immunoreactivity is present within the stroma or epithelium. There is some immunostaining present within the leukocyte population in the vessel shown with *. (C) Intense immunoreactivity is present in the epithelium and is diffuse throughout the decidualised stromal cells. (D) Immunostaining is present in the glandular epithelium and secretions. Scale bars = 100 µm.

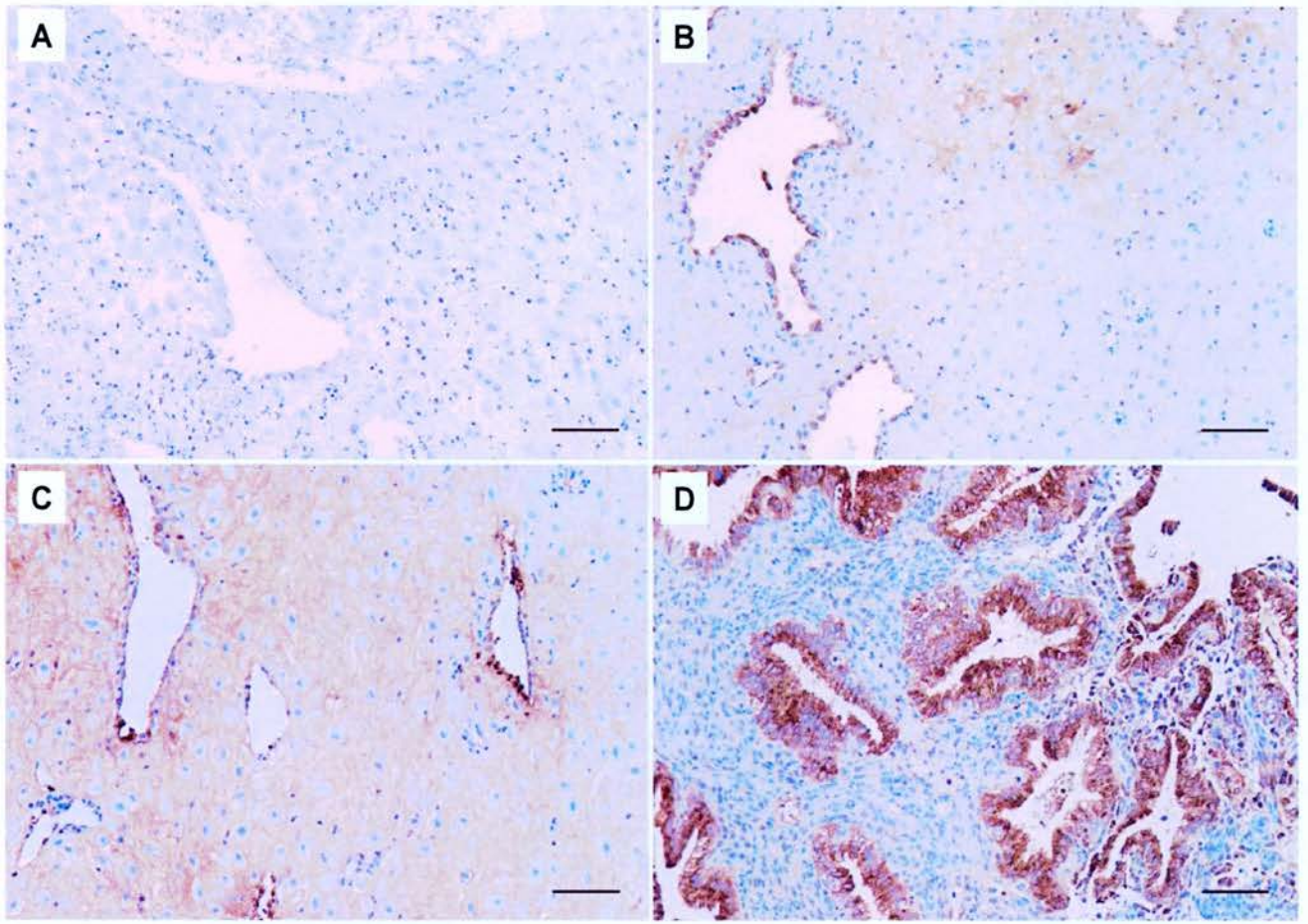


Figure 7.3.2.2 Immunohistochemical localisation of SLPI in human decidua obtained from STOP (B), miscarriage (C) and ectopic gestation (A&D). (A). Negative control (decidua from STOP). Primary antibody replaced with an equimolar concentration of mouse immunoglobulin. (B) Immunoreactivity is present within the glandular epithelium. There is some immunostaining present within the stroma. (C) Intense immunoreactivity is present in the epithelium and is diffuse throughout the decidualised stroma. The cytoplasm and nucleus of the stromal cells do not exhibit immunoreactivity. (D) Intense immunoreactivity was observed in the glandular epithelium . Scale bars = 100 μm.

7.4 Discussion

The decidua obtained from women with surgical management of miscarriage, termination of pregnancy and tubular ectopic pregnancies have been investigated for the expression of natural antimicrobials and MMP-7. The decidua obtained from women with miscarriage has been included as an 'abnormal' control in order to try and elucidate the difference in the expression of these genes over what might be expected from an inflammatory response and with the inclusion of first trimester decidua (STOP) as 'normal' controls.

Elafin and MMP-7 mRNA levels were found to be increased in the decidua obtained from women with an ectopic gestation when compared to the decidua from women undergoing STOP and with miscarriage. The increase in elafin mRNA in the decidua from ectopic pregnancy is 65-point greater than the decidua of women with ongoing intra-uterine pregnancy, and 60-point greater than the decidua from women with miscarriage patients. There may be a slight increase in the level of elafin and MMP-7 mRNA in the decidua obtained from miscarriage. The data obtained from the decidua of both ectopic gestation and STOP is similar to the data obtained from the corresponding Fallopian tube biopsies presented in chapter 6.

There was no difference observed for the level of SLPI mRNA between the decidua obtained from women with a STOP and women with miscarriage. However, there was a marked increase in the level of SLPI mRNA in decidua from women with an ectopic pregnancy; this was also seen in the Fallopian tube with an ectopic gestation (chapter 6). SLPI mRNA has been previously shown to be present during the first

trimester of pregnancy and is responsive to progesterone ((King, Critchley et al. 2000; Fleming, King et al. 2003; King, Morgan et al. 2003) and chapter 5).

The results obtained from the analysis of the decidua obtained from women with an ectopic pregnancy appear to match the levels observed in the corresponding Fallopian tube biopsies (chapter 6). Thus, it may be conceivable that the use of endometrial biopsies could prove helpful in the early diagnosis of ectopic pregnancy. Previous studies investigating the use of frozen Pipelle endometrial biopsies as a means of diagnosis for an ectopic pregnancy have proved to be unsuccessful (Al-Ramahi, Nimri et al. 2006). However, it has been shown herein that the use of Pipelle endometrial biopsies with an analysis of the level of natural antimicrobials and MMP-7, that an ectopic gestation may be distinguished from a normal intra-uterine pregnancy (STOP). However, the numbers need to be increased before conclusive discussion and further clinical studies would be warranted. Furthermore, it can be surmised that the increase in both natural antimicrobials and MMP-7 is also distinguishable between an ectopic gestation and that of an inflammatory uterine pregnancy (miscarriage). An intrauterine abortion (miscarriage) must also be distinguished from an ectopic pregnancy and further methods for an accurate differential diagnosis between these complications have been sought (Nyberg, Filly et al. 1987; Mashburn 1999). A number of studies have disputed the reliability of circulating progesterone levels in differentiating between an ectopic pregnancy and a miscarriage (McCord, Muram et al. 1996; O'Leary, Nichols et al. 1996; Dart, Ramanujam et al. 2002). Further work is required in order to fully understand the role of natural antimicrobials and MMP-7 in gestational complications. The role of

infection would need to be taken into account as the present study did not attempt to discriminate between subjects with a history of infection or pelvic inflammatory disease, which as well as being a risk factor for an ectopic pregnancy would also upregulate expression of the natural antimicrobials. Thus, the present study also does enable the determination between cause and effect as it is conceivable that the increased presence of these molecules could have a causal role in ectopic pregnancy.

An increased level of MMP-7 for example, has been associated with excess damage to the extracellular matrix (ECM). MMP-7 is upregulated in the event of injury or infection (Lopez-Boado, Wilson et al. 2000; Lopez-Boado, Wilson et al. 2001). MMP-7 is hypothesised to have a role in immune defence and thus, a role in the regulation of the natural antimicrobials is possible. Unlike most of the other MMPs there has yet to be a substrate identified for MMP-7 (Pal, Schmidt et al. 2006). MMPs have also been associated with the pathogenesis of *Chlamydia trachomatis,* a sexually transmitted infection. The long term implications of an infection with *C. trachomatis* is the build up scar tissue for which the MMPs are thought to be responsible (Pal, Schmidt et al. 2006). This scar formation in the Fallopian tube is also believed to be a cause of ectopic pregnancy (Stamm 2001; Andersen, Ostergaard et al. 2007). However, the histological examination of Fallopian tubes with a history of infection with *Chlamydia* often shows no evidence of scarring (communication from Dr Alistair Williams).

The human β-defensins have also been found to be differentially expressed in each of the decidual groupings, with the exception of hBD1, the defensins are greatly increased in decidua with an ectopic gestation. Human β-defensin 1 mRNA levels are at a similar level in decidua with miscarriage and STOP. This suggests that miscarriage does not involve an increase in the level of hBD1 in comparison with normal first trimester pregnancy. However, there is almost a 50% relative decrease in the level of hBD1 mRNA (not significant) in decidua from women with an ectopic pregnancy compared to the STOP and miscarriage. The inhibition of hBD1 expression in decidua from ectopic gestation may be an indication of the presence of inhibitory factors. Further investigation would be required to address questions such as, what factors/mechanisms could be involved in this inhibition and is the decrease in expression of this defensin representative of a causal or contributory role with regard to an ectopic outcome? Human β-defensin 1 has been shown to be constitutively expressed in the cycling endometrium and in the first trimester of pregnancy (Fleming, King et al. 2003; King, Critchley et al. 2003). Furthermore, it has been shown that unlike most of the other defensins, hBD1 is only very slightly upregulated in the presence of inflammatory stimuli (Bajaj-Elliott, Fedeli et al. 2002). There may be an unidentified role for hBD1 in pregnancy and this decrease in decidua obtained from women with an ectopic gestation may be indicative of a change in conditions favourable to the expression of hBD1 and indeed pregnancy. Investigation of hBD1 expression in the Fallopian tube with and without an ectopic gestation demonstrated no change in the level of expression (chapter 6).

Messenger mRNA levels for hBD2 are relatively increased in decidua from miscarriage subjects and further increased in decidua from women with an ectopic gestation when compared to decidua from women having a STOP (not significant). Human β-defensin 2 is found to be expressed in a variety of different mucosal linings such as the lung, and is inducible by inflammatory stimuli (Liu, Destoumieux et al. 2002; Sorensen, Cowland et al. 2003; Tsutsumi-Ishii and Nagaoka 2003) including Hec-1A endometrial epithelial cells (chapter 3). It has also been proposed that hBD2 may be inhibited directly or indirectly by progesterone (chapter 3; (Fleming, King et al. 2003) and thus, elevation in early pregnancy would not be predicted. This could be suggestive of a problem with progesterone levels or with progesterone mediated factors, giving rise to increased levels of hBD2 in decidua of miscarriage and ectopic gestation. Human β-defensin 2 mRNA also has a tendancy to be increased in Fallopian tubes with an ectopic pregnancy when compared with Fallopian tubes without an ectopic gestation (chapter 6).

The level of hBD3 are also increased in decidua from women with an ectopic pregnancy and is almost 350-point greater than decidua from women having STOP (figure 7.3.1.3 (D); $P<0.05$). In contrast the expression of hBD3 was unchanged between the decidua from patient having STOP and with miscarriage subjects. Human β-defensin 3 is maximally expressed during the secretory phase of the menstrual cycle (King, Fleming et al. 2003). However, the treatment of primary or cultured epithelial cell lines with progesterone did not evoke a change in expression (King, Fleming et al. 2003). The increase in hBD3 mRNA in decidua from women with an ectopic gestation may be an indicator of inflammation as hBD3 is associated

with the attraction of macrophages (Yang, Chertov et al. 1999), and monocytes (Garcia, Jaumann et al. 2001). In chapter 6, data were presented that demonstrated that there was also an increase in the level of hBD3 mRNA in Fallopian tube biopsies with an ectopic gestation (chapter 6; figure 6.2.5.1A).

There is a tendancy for an increase in the level of hBD4 in decidua from women with miscarriage and a further increase in decidua from patients with an ectopic gestation when compared to decidua from women having STOP (not significant). This defensin has been previously described as being maximally expressed during the proliferative phase of the endometrium and has not been reported to respond to inflammatory cytokines or mimics of infection (King, Fleming et al. 2003). The expression of this gene may be oestradiol mediated as shown both in primary endometrial samples (King, Fleming et al. 2003) and in the Fallopian tube biopsies presented in chapter 6. The expression of hBD4 in decidua from women with an ectopic suggestion may therefore be representative of aberrant steroid levels. The Fallopian tube biopsies with an ectopic gestation were also shown to have an increased level of hBD4 mRNA (chapter 6; figure 6.2.5.1A). Once again it has been demonstrated that an elevated level of hBD4 in decidua is representative of an elevated level within the Fallopian tube with an ectopic gestation and thus, may be a useful candidate for a diagnostic marker.

In the previous chapter it was demonstrated that a pattern of expression existed between SLPI, elafin and MMP-7 (chapter 6; figure 6.2.5.2), in the Fallopian tube with and without an ectopic gestation. Thus, the expression of these molecules

within the decidua from subjects having STOP, miscarriage and surgical treatment of ectopic pregnancy were similarly examined (figure 7.3.1.2). As with the data presented in chapter 6 there is a suggestion of a possible relationship between SLPI, elafin and MMP-7, and that this was disrupted in both decidua from women with miscarriage and women with an ectopic gestation. The levels of elafin and MMP-7 mRNA levels were found to be greatly upregulated in decidua from women with an ectopic gestation, and there was also a doubling in the level of SLPI mRNA. In decidua from STOP (B) all except one of the biopsies exhibits an increased level of SLPI and lower levels of elafin and MMP-7 and previous work has confirmed that SLPI is present during the first trimester of pregnancy (King, Critchley et al. 2000). The inhibition of both elafin (chapter 3) and MMP-7 (Bruner, Rodgers et al. 1995) in the presence of progesterone has also been suggested previously and these molecules are not thought to be expressed during the first trimester of pregnancy. Therefore it may be that the increased expression of these molecules in decidua obtained from women with miscarriage or with ectopic pregnancy are indications of some aberration in progesterone mediated mechanisms. The decidua from women with miscarriage demonstrates a similar level of SLPI mRNA expression when compared to decidua from women with a STOP. However, as with the decidua with an ectopic gestation, there was increased expression of both elafin and MMP-7 mRNA, but, lower than in decidua with an ectopic gestation.

7.4.1 Future work

The potential for natural antimicrobials and MMP-7 to act as diagnostic markers in the decidua obtained from patient groups where a risk of an ectopic is indicated had been supported with the data presented and merits further investigation. The inclusion of data relating to subjects with current or past infection with sexually transmitted infections, such as chlamydia, may help to further understand the expression levels of natural antimicrobials and MMP-7. Further work is also required in the localisation and the quantitative analysis of variable levels of protein in tissue biopsies, which was not possible within this study due to time constraints. An investigation into the role of MMP-7 in decidua of normal and abnormal early pregnancy pathology is also merited and further consideration as to the interaction with elafin, direct, indirect or coincidental. Some further investigation into the possible relationship between elafin, MMP-7 and SLPI is presented in Chapter 8 of the current thesis.

Chapter 8:

An investigation into the potential relationship between SLPI, elafin and MMP-7 in Hec-1A cells.

Chapter 8: An investigation into the potential relationship between SLPI, elafin and MMP-7 in Hec-1A cells.

8.1 Introduction

SLPI (secretory leukocyte protease inhibitor) and elafin are both members of the serine antiprotease family (Sallenave 2000; Hiemstra 2002; Sallenave 2002). These proteins have been identified as alarm proteinase inhibitors as they are rapidly upregulated in the event of injury or infection. In response to inflammation serine proteases are produced such as human neutrophil elastase (HNE) by immune cells and are responsible for a number of inflammatory disorders and exert proteolytic activity which can result in tissue degradation (Sallenave 2000; Hiemstra 2002; Sallenave 2002). Thus, it is believed that the role of SLPI and elafin is to prevent the over exuberance of the proteolytic response and to minimise tissue damage. The balance between proteases and protease inhibitors is important to the maintenance of tissue integrity (Sallenave 2000; Hiemstra 2002; Sallenave 2002). In addition to the anti-protease activity of both, SLPI and elafin exhibit chemotactic and antimicrobial activity, similar to that observed in the defensins (Hiemstra, Fernie-King et al. 2004). As discussed throughout the current thesis both elafin and SLPI are upregulated by inflammatory stimuli such as the pro-inflammatory cytokines, TNFα and IL-1β and mimics of infection such as LPS. The regulation of elafin expression via inflammatory stimuli has been reported to be mediated at the transcriptional level by the transcription factor nuclear factor κ B (NF-κB) (Bingle, Tetley et al. 2001) and the transcription factor activating protein-1 (AP-1) (Zhang, Magit et al. 1997). SLPI has been shown to act as an anti-inflammatory with the inhibition of HIV infection of monocytes (McNeely, Shugars et al. 1997). SLPI has further been demonstrated to

inhibit the LPS induced expression of MMP-9 and TNFα in monocytic cells (Jin, Nathan et al. 1997; Zhang, DeWitt et al. 1997). In the rat lung, SLPI has been shown to prevent activation of NF-κB in response to stimulation with IgG immune complexes (Lentsch, Jordan et al. 1999). SLPI can mediate further anti-inflammatory action via the up-regulation of other anti-inflammatory molecules such as IL-10, TGF-β and HGF (hepatocyte growth factor) (Kikuchi, Abe et al. 2000; Sano, Shimizu et al. 2000).

SLPI and elafin have been identified as being multi-functional members of the immune defence with other roles and interactions still to be fully elucidated. In this thesis a number of questions were raised as result of investigations into the function of these proteins in the context of the female reproductive tract and the current chapter was written in response to these observations. Therefore the following summary of the relevant findings contained within this thesis as a motivation to further work was deemed necessary.

Chapter 3

In chapter 3, an examination into the differential timing of mRNA expression of natural antimicrobials in the endometrial epithelial cell line Hec-1A, seemed to suggest that both SLPI and elafin were expressed in a reciprocal pattern (figure 3.4.2.1). The maximal expression of SLPI mRNA coincided with a lower level of elafin mRNA and vice versa. This occurred consistently between experiments and at

different time points within the timecourse experiments discussed in chapter 3 of the current thesis. This raised the possibility that there may be a mechanism of direct or indirect interaction between these molecules.

Chapter 4

An investigation into the role of paracrine mediated factors in the expression of elafin in the endometrium was undertaken in chapter 4. This was in response to the differential expression levels of elafin in the presence of progesterone and this was suggested to be as a result of a stromal mediated effect upon the epithelial cells. An examination of the literature identified possible factors that could be responsible for the mediation of epithelial elafin expression via the stroma in response to progesterone. The main candidate was considered to be TGFβ-1, as this has been shown to be upregulated by the stroma in response to progesterone (Bruner, Rodgers et al. 1995) and within this thesis to inhibit elafin (figure 3.4.3.1 and 3.4.3.2) and has been previously reported to inhibit the expression of SLPI (Jaumann, Elssner et al. 2000). It was also shown that TGFβ-1 expression by the stroma may mediate epithelial expression of MMP-7, thus, this was investigated further for a possible role in the mediation of natural antimicrobial expression within the female reproductive tract. Matrix metalloproteinase-7 has been similarly identified as an important mediator of immune defense with an initial characterised role in injury and repair (Lopez-Boado, Wilson et al. 2000). MMP-7 is constitutively expressed across a number of mucosal surfaces and is up-regulated in response to injury or infection in much the same manner as components of the innate immune defence (Lopez-Boado,

Wilson et al. 2000). MMP-7 has been identified as being involved in the degradation or processing of other proteins, such as elastin (Filippov, Caras et al. 2003). In the murine paneth cells, MMP-7 has been shown to be responsible for the cleavage of pro-defensins into the active form and that mice without MMP-7 demonstrated a reduced capacity for bacterial clearance (Wilson, Ouellette et al. 1999; Ayabe, Satchell et al. 2002). Elafin and the human β-defensins have also been found to exist in a pre-mature form that can be spliced for activity or for different functionality (Schalkwijk, Wiedow et al. 1999; Guyot, Zani et al. 2005; Pazgier, Hoover et al. 2006). However, there is little conclusive evidence for the factor(s) or mechanism(s) involved and there are suggestions that such interactions may be site specific. Thus, MMP-7 could be a possible candidate within the female reproductive tract and as such was examined within the current thesis. The cDNA obtained in chapter 3 for the analysis of antimicrobial expression across a timecourse was reanalysed for MMP-7 mRNA expression and the pattern compared to elafin (figure 8.1.1). The mRNA expression pattern of MMP-7 over time was similar to that obtained for elafin, like elafin MMP-7 exhibits two major peaks of expression each with a subsequent decline. However, the maximal expression peaks for MMP-7 occurs in a 'delayed' fashion. Elafin peaks at 8 hour and declines at 10 hour, whilst MMP-7 peaks at 10 hour and declines at 12 hour and similar was seen for subsequent time points. This delay may be suggestive of a processing role for MMP-7 on the pre-elafin molecule. Alternatively, MMP-7 may serve to degrade elafin to prevent an over exuberant inflammatory response, perhaps the phasic expression of elafin is due to the degradation and re-expression of elafin.

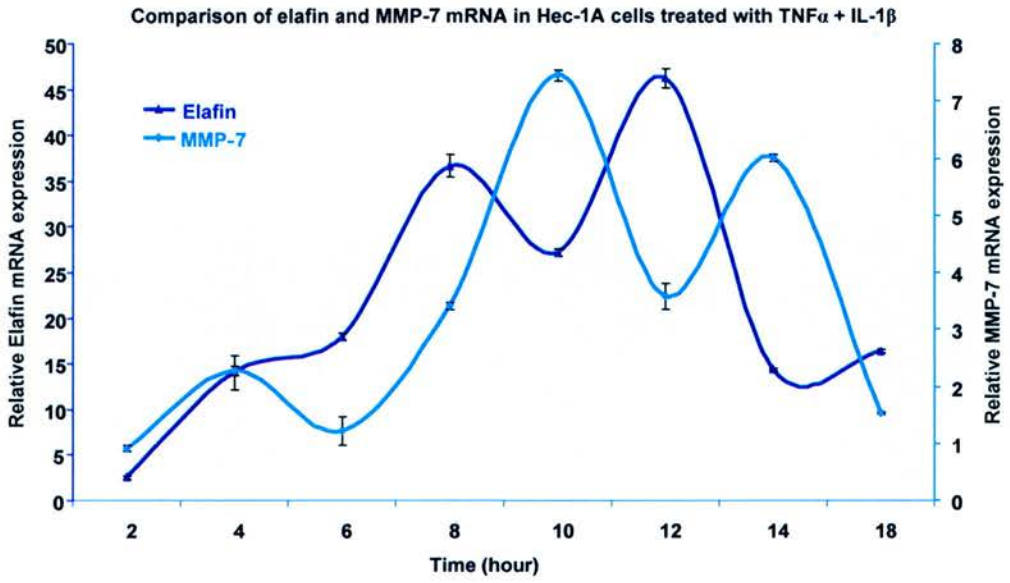


Figure 8.1.1 Elafin (dark blue) and MMP-7 (light blue) mRNA expression over time plotted on two axes on different scales. The left axis is representative of elafin levels and the right axis MMP-7. The data were obtained as described in chapter 3 figure 3.4.1.1, of the current thesis.

Chapter 5

The pattern of SLPI, elafin and MMP-7 mRNA expression in primary endometrial biopsy material was re-examined in chapter 5 and is summarised below (figure 8.1.2). The data obtained seemed to lend further support to an 'antagonistic' relationship between elafin and SLPI as their mRNA expression occurs at different times during the menstrual cycle. It was also observed that MMP-7 was expressed along with elafin during the menstrual phase and in the subsequent proliferative phase, which may be representative of the delay observed during the timecourse in figure 8.1.1.

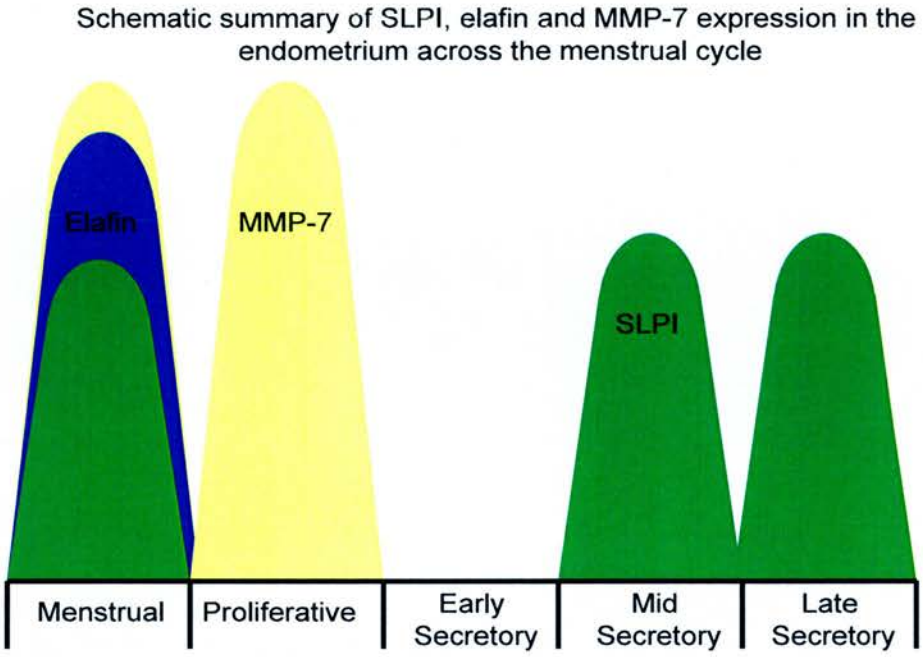


Figure 8.1.2 Schematic summary of the data presented in chapter 5; figures 5.3.2.1 (elafin), 5.3.3.1 (SLPI) and 5.3.4.1 (MMP-7)

Chapter 6

In chapter 6 the expression of SLPI, elafin and MMP-7 was investigated in biopsy (Fallopian tube) samples obtained from woman with and without an ectopic gestation (figure 6.2.5.2). An interesting pattern of expression could be observed in the individual samples without an ectopic gestation (figure 8.1.3). It was found that a sample would demonstrate one of two possible expression patterns. In pattern one, the expression of SLPI mRNA was found to be lower than the level of both MMP-7 and elafin and this was true of 6 of the 11 samples examined. In pattern two, the expression of SLPI mRNA was found to be greater than both elafin and MMP-7 mRNA (4 out of 11). It was also observed that elafin expression was higher than MMP-7 mRNA in the majority of the patient samples.

In Fallopian tube biopsy samples collected from women with an ectopic gestation there was an increase in both elafin and MMP-7 mRNA in 7 out of the 9 patients sampled. There was only a small increase in the level of SLPI mRNA in the same samples. These patient samples also exhibited a change in the expression between elafin and MMP-7. The level of MMP-7 was found to be higher than the level of elafin, which is the opposite of what was observed previously in patients without an ectopic pregnancy.

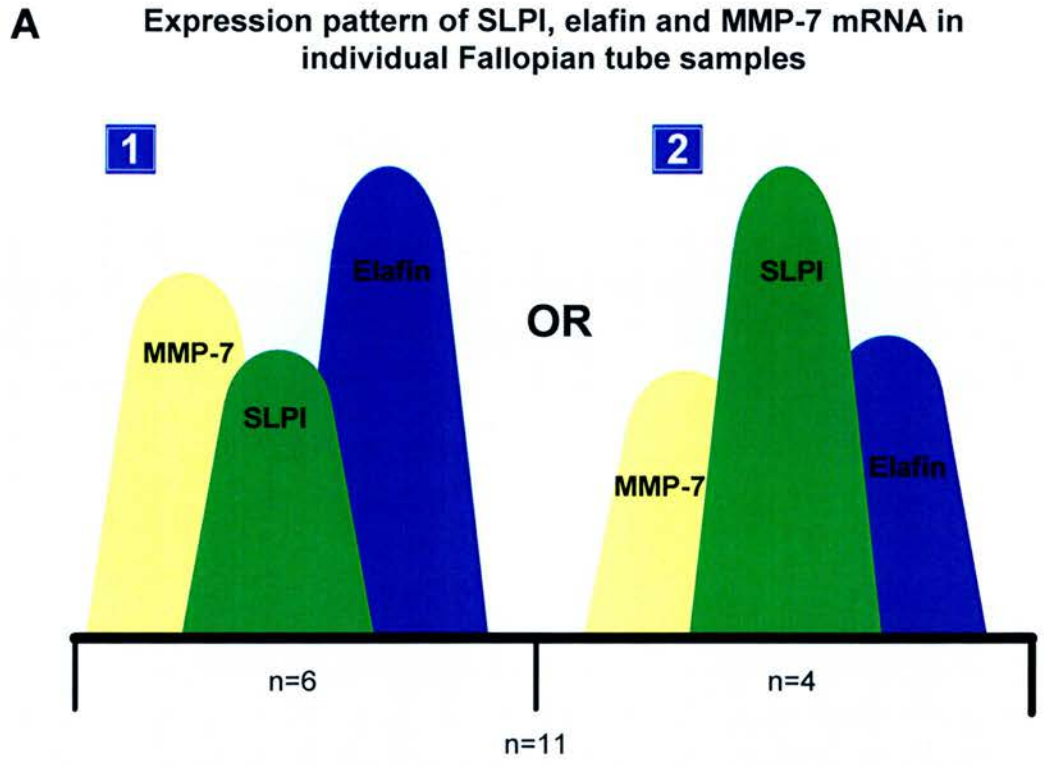


Figure 8.1.3 A cartoon representation of the data presented in chapter 6 (figure 6.3.2.2) of the current thesis. The expression pattern of SLPI (green), elafin (blue) and MMP-7 (yellow) in relation to one another within individual Fallopian tube samples, which presented as one of the two outcomes depicted in 10 of the samples examined (n=11). Pattern one with the level of SLPI mRNA lower than both elafin and MMP-7 levels. Pattern two, the level of SLPI mRNA expression was higher than the levels of elafin and MMP-7 mRNA.

Chapter 7

There were similar findings in chapter 7 when the expression of these three molecules was investigated in decidua collected from women undergoing a surgical termination of pregnancy. The pattern was predominantly as shown on the right hand side (1) of the above cartoon (figure 8.1.3) with only one sample demonstrating the pattern depicted on the left (2) (n=5).

The observations throughout this thesis that there is a likely relationship exists between SLPI, elafin and MMP-7 expression, has prompted the investigations undertaken in the current chapter with the following aims.

- To investigate the effect of SLPI upon the expression of elafin and MMP-7 using the cultured cell line Hec-1A.
- To explore the effects of SLPI upon other innate immune effectors including the β-defensins and cytokines.
- To examine any effect of MMP-7 upon the expression of elafin
- To investigate whether SLPI has a direct immunomodulatory role over elafin by binding to the promoter of elafin.

8.2 Methods

8.2.1 Cell culture

The Hec-1A endometrial epithelial cells were cultured as described in 2.2.3. The cells were seeded into 6 well plates prior to treatment. The treatments used in this chapter are detailed in table 8.2.1.1.

Treatment	Concentration used
Control	n/a
IL-1β	5 ng/ml
TNFα	5 μg/ml
SLPI	5 ng/ml
MMP-7	5 ng/ml

Table 8.2.1.1 Details of treatments used within this chapter. Supplier information is contained within appendix I.

8.2.2 Transfection (transient)

The plasmid DNA was prepared as detailed in section 2.5.1 and the constructs are shown in figure 8.2.2.1. The transfection efficiency was optimised and the appropriate ratio of reagent (FuGene HD) to DNA determined using the β-galactosidase assay detailed in section 2.5.2.

The Hec-1A cells were grown to 70% confluence in 6-wells and transfected with 5 μg of CAT reporter vectors driven by the elafin promoter or the control promoter as described in section 2.5.3. Cells were stimulated with TNFα (5 μg/ml) + IL-1β (5

ng/ml), 8 hour after transfection and 20 hour before cell lysis. CAT production was measured by ELISA (Chapter 2, section 2.4.3).

8.2.3 RNA extraction and Q-RT-PCR

Following treatment and incubation for the relevant time point, the RNA was extracted from the cells and cDNA prepared as described in chapter 2, section 2.3. Elafin mRNA levels were measured in these cDNA samples by quantitative PCR (chapter 2, section 2.3). The sequence details of the primer-probe sets used are detailed in table 2.3.3.1, materials and methods.

8.2.4 CAT ELISA

The sandwich ELISA quantitatively measures the amount of CAT enzyme expression as mediated via the elafin promoter in response to treatment as detailed in 8.2.2. The ELISA was carried out according to the manufacturer and is described in chapter 2, section 2.4.3).

8.2.5 Statistical analysis

The PCR results in this chapter were analysed by ANOVA for significant difference.

8.2.6 Acknowledgement

The transient transfections and the CAT ELISA assays were carried out with the assistance of Dr Forbes Howie and Dr Moira Nicol of the University of Edinburgh, Department of Clinical Biochemistry.

8.3 Results

8.3.1 The effect of SLPI upon Hec-1A mRNA expression of elafin and MMP-7 in response to treatment with inflammatory stimuli TNFα + IL-1β.

In order to further understand the effect of SLPI upon the mRNA expression of both elafin and MMP-7, the protein (SLPI 5 ng/ml) was added to Hec-1A cells in culture along with inflammatory stimuli (TNFα + IL-1β).

Elafin mRNA (figure 8.3.1.1) expression levels were decreased in response to treatment with TNFα + IL-1β in the presence of SLPI up to 18 hour post treatment (A) when compared to the expression in the absence of SLPI (n=3). Elafin mRNA (B) was significantly ($P<0.001$) decreased in the presence of SLPI when time points 4, 8 and 12 hour were averaged for each n=1 and a total of 5 times (n=5). There was no notable effect observed from the treatment of Hec-1A cells with SLPI without inflammatory stimuli (controls).

MMP-7 mRNA (figure 8.3.1.2) expression levels were also found to be significantly decreased with the addition of SLPI (5 ng/ml) in Hec-1A cells treated with TNFα + IL-1β (n=5; $P<0.001$). The addition of MMP-7 to untreated cells consistently resulted in a lowering of the 'basal' levels of elafin mRNA when compared to cells untreated and without MMP-7 (n=5).

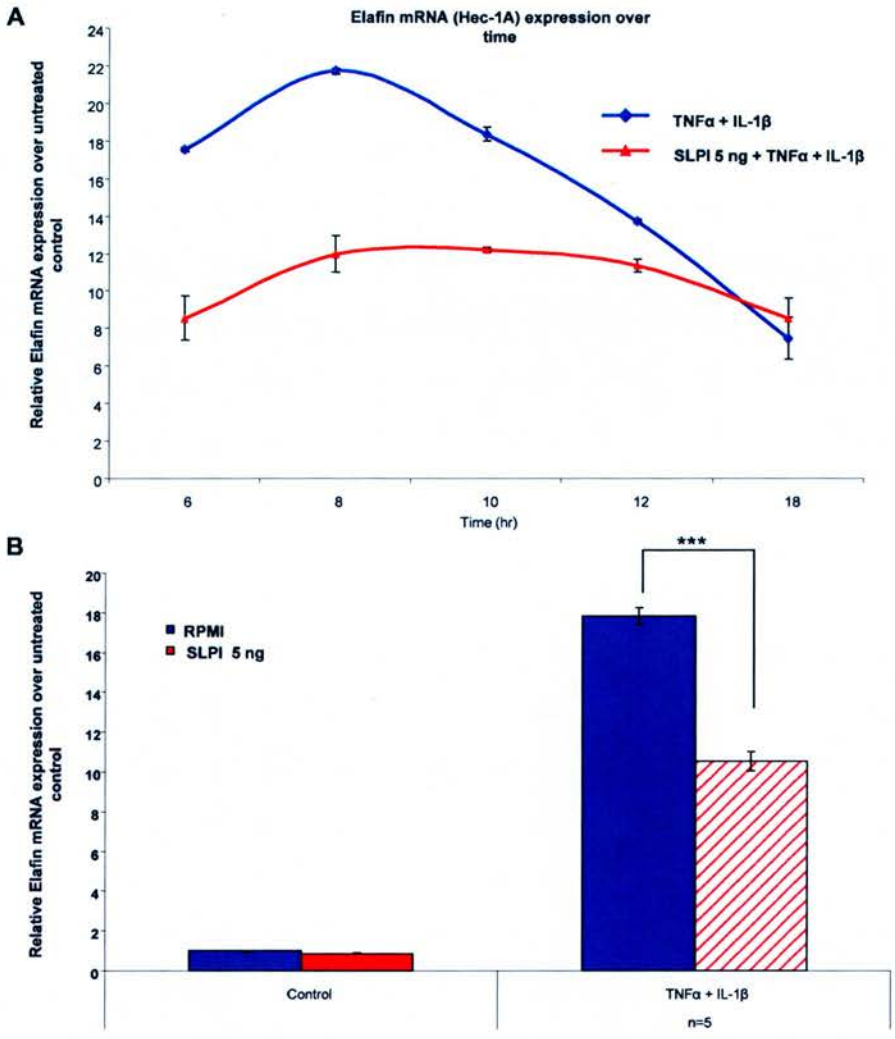


Figure 8.3.1.1 The effect of SLPI protein (5 ng/ml) upon the expression of elafin in response to inflammatory stimuli, TNFα + IL-1β The expression of elafin over time (A) with (red) and without (blue) the presence of SLPI. Elafin mRNA presented as a mean of data collected from 3 time points (4, 8 and 12 hour equivalent to n=1), is significantly decreased (n= 5; $P<0.001$) in the presence of SLPI. All data are compared to untreated Hec-1A cells from the relevant time point as control, given a nominal value of 1, mean ± s.e.m.

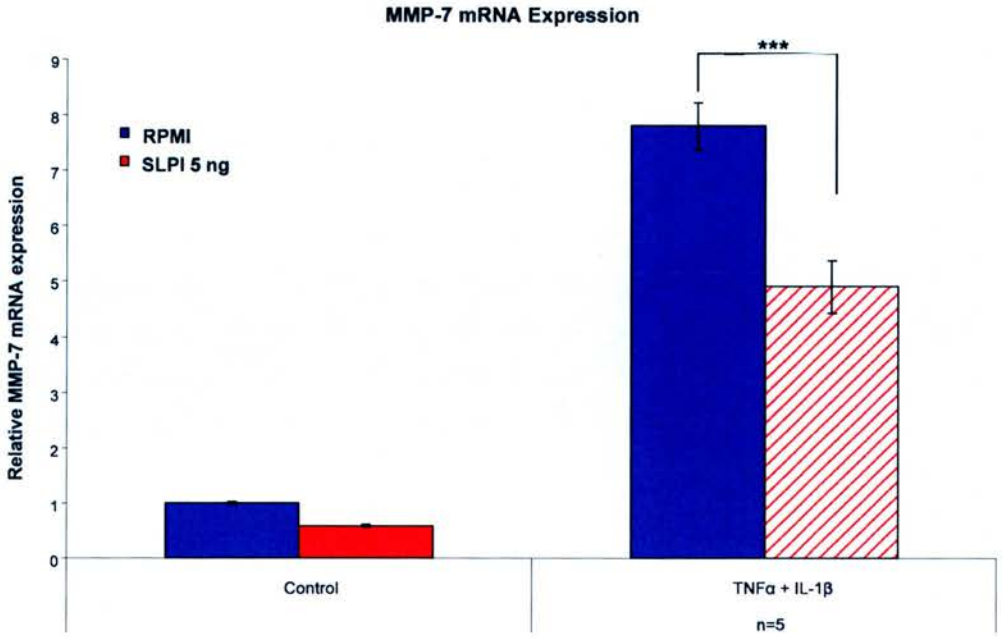


Figure 8.3.1.2 The effect of SLPI protein (5 ng/ml) upon the expression of MMP-7 in response to inflammatory stimuli, TNFα + IL-1β. MMP-7 mRNA presented as a mean of data collected from 3 time points (4, 8 and 12 hour equivalent to n=1), is significantly decreased (n= 5; $P<0.001$) in the presence of SLPI. All data are compared to untreated Hec-1A cells from the relevant time point as control, given a nominal value of 1, mean ± s.e.m.

8.3.2 The effect of SLPI on SLPI, hBD2 and hBD3 mRNA expression in Hec-1A cells treated with TNFα + IL-1β.

In order to examine whether the 'anti-inflammatory' nature of SLPI was general an examination of the effect upon the expression of the β-defensins and that of SLPI in Hec-1A cells stimulated with pro-inflammatory cytokines TNFα + IL-1β.

SLPI mRNA (figure 8.3.2.1 (A)) was observed to be increased in response to treatment with SLPI protein (green), (5 ng/ml). This increase was significant in cells with and without the concurrent treatment with TNFα + IL-1β, when compared to the cells without the presence of SLPI (blue) ($n=5$; $P<0.001$).

Messenger RNA expression of human β-defensin 2 (figure 8.3.2.1 (B)) was similarly found to be significantly increased in the presence of SLPI protein in response to treatment with TNFα + IL-1β ($P<0.001$).

Human β-defensin 3 mRNA (figure 8.3.2.1 (C)) was found to be increased in response to the presence of SLPI both with and without the treatment with TNFα + IL-1β. There was no significance between the cells with and without TNFα + IL-1β in the presence of SLPI (green; TNFα + IL-1β, green stripe). The expression of hBD3 is significantly increased with SLPI alone relative to the untreated control. A 2-point increase in hBD3 mRNA was observed in cells treated with TNFα + IL-1β in the presence of SLPI compared to without SLPI.

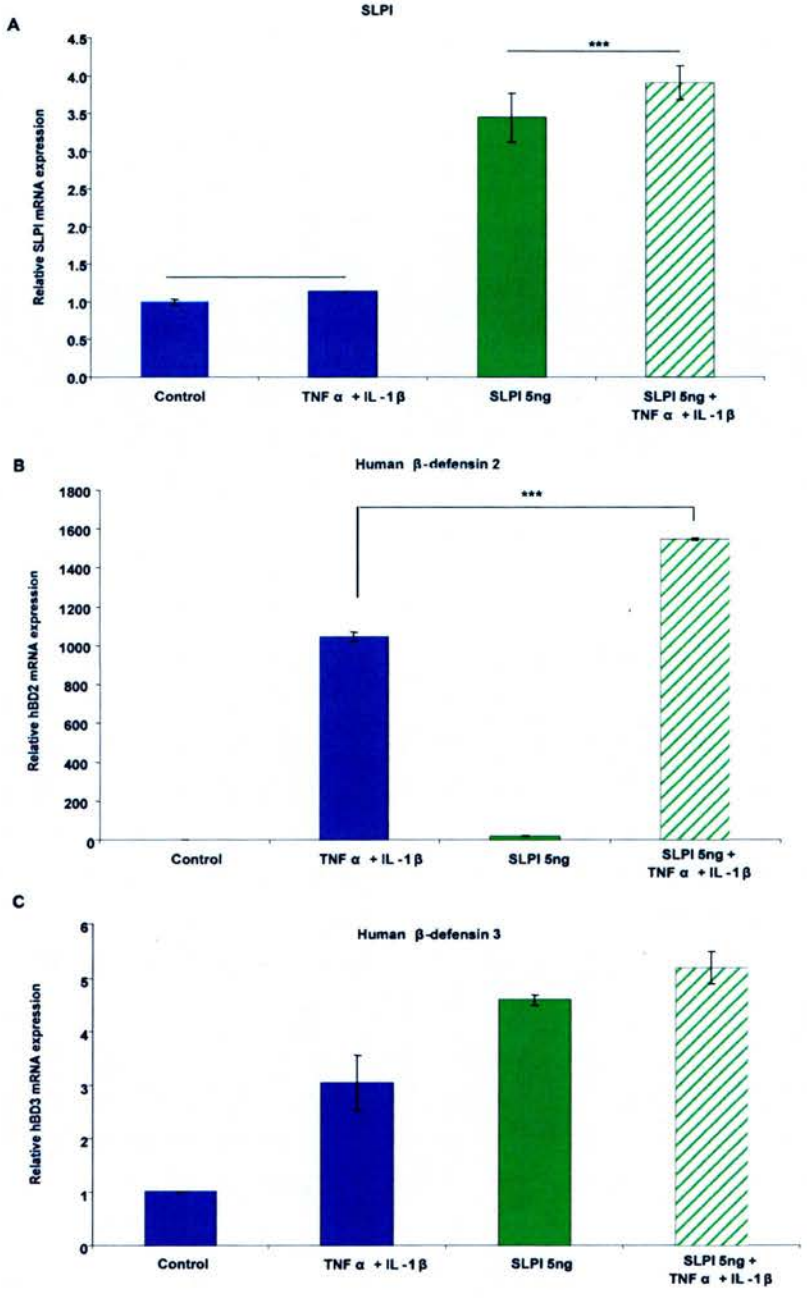


Figure 8.3.2.1 The effect of SLPI protein (5 ng/ml) on the mRNA expression of SLPI (A), hBD2 (B) and hBD3 (C) in response to inflammatory stimuli, TNFα + IL-1β. Data was collected from 3 time points (4, 8 and 12 hour equivalent to n=1). All data were compared to untreated Hec-1A cells from the relevant time point as control, given a nominal value of 1, mean ± s.e.m.

8.3.3 Hec-1A expression of cytokines, TNFα, IL-1β, IL-8 and IL-6 in the presence of SLPI.

Further to the data obtained in section 8.3.1 which indicated that SLPI had an inhibitory effect upon the expression of elafin and MMP-7, which was in contrast to the data obtained in section 8.3.2 where SLPI was shown to increase the expression of human β-defensins and SLPI mRNA. It was decided to examine the possibility of indirect effects via the interaction with cytokines and thus the effect of SLPI protein upon the Hec-1A expression of cytokines was examined.

The expression of TNFα mRNA, figure 8.3.3.1 (A), was significantly increased in response to treatment with the pro-inflammatory cytokines TNFα + IL-1β in the presence of SLPI ($n=4$; $P<0.001$) when compared to cells treated without SLPI. There was a small increase in the level of TNFα mRNA in response to SLPI without inflammatory stimuli when compared to cells in the absence of SLPI.

Messenger RNA expression of IL-1β, figure 8.3.3.1 (B), was increased in the presence of SLPI and inflammatory stimuli ($n=4$; $P<0.05$) in comparison to treatment with TNFα + IL-1β without SLPI.

In order to further establish the role of SLPI as a possible anti-inflammatory mediator it was deemed necessary to explore the effect upon other key innate immune effectors such as the pro-inflammatory cytokines IL-6 and IL-8.

In contrast to the observed increase of TNFα and IL-1β in the presence of SLPI an examination of IL-8 and IL-6, cytokines which are also associated with pro-inflammatory signalling were found to be decreased by the addition of SLPI.

Interleukin – 8 mRNA, figure 8.3.3.2 (A), was significantly decreased (n=4; $P<0.001$), when treated with TNFα + IL-1β in the presence of SLPI when compared to treatment without SLPI (25-point). However, a small increase in the level of IL-8 mRNA was observed in cells treated with SLPI alone.

The level of IL-6 mRNA, figure 8.3.3.2 (B), was also decreased in response to treatment with TNFα + IL-1β in the presence of SLPI (n=4; $P<0.001$). A 50-point reduction could be observed between cells treated with TNFα + IL-1β without SLPI and those that were treated with the addition of SLPI.

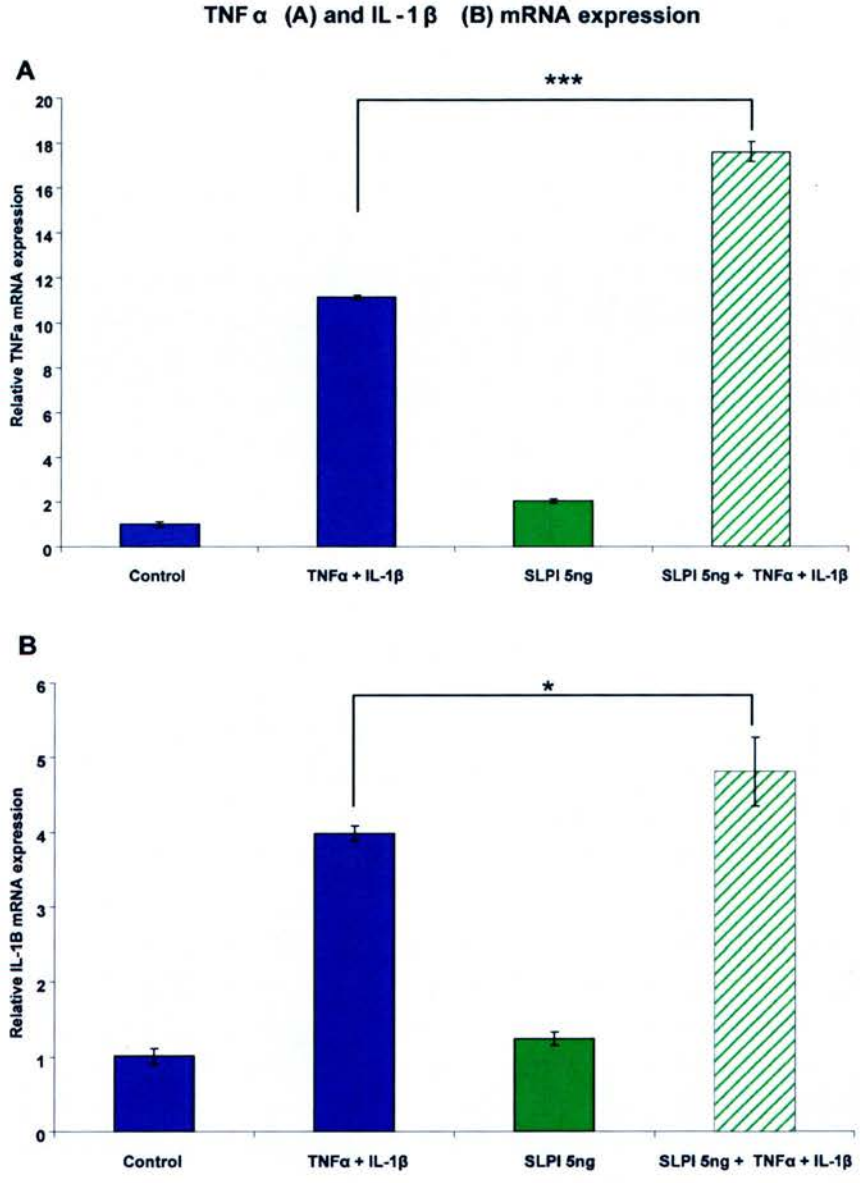


Figure 8.3.3.1 The effect of SLPI protein (5 ng/ml) on the mRNA expression of TNFα (A) and IL-1β (B) in response to inflammatory stimuli, TNFα + IL-1β (n=4). The mRNA levels of both TNFα and IL-1β were increased in the presence of SLPI. The un-stimulated control was also increased with the addition of SLPI. Data was collected from 3 time points (4, 8 and 12 hour equivalent to n=1). All data were compared to untreated Hec-1A cells (control) from the relevant time point as control, given a nominal value of 1, mean ± s.e.m.

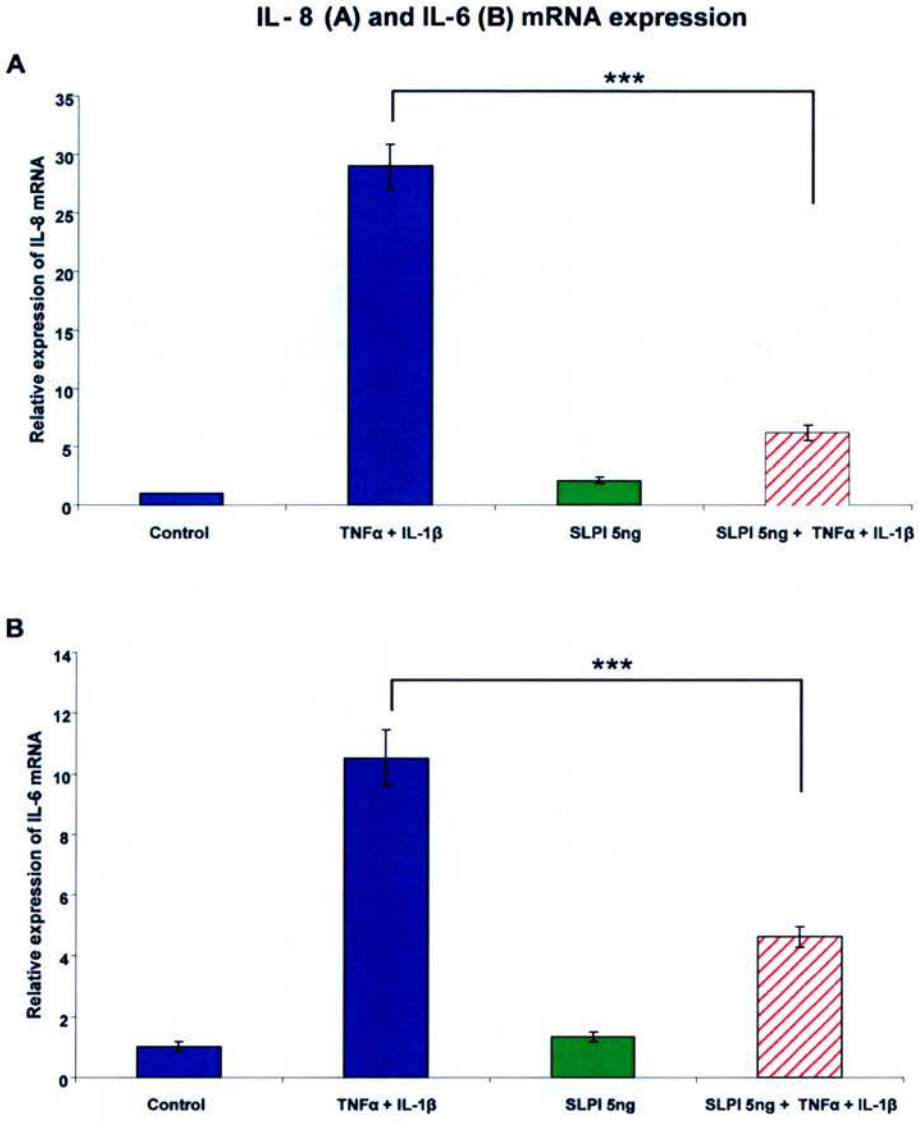


Figure 8.3.3.2 The effect of SLPI protein (5 ng/ml) on the mRNA expression of IL-8 (A) and IL-6 (B) in response to inflammatory stimuli, TNFα + IL-1β (n=4). The mRNA levels of both IL-6 and IL-8 were decreased in the presence of SLPI. The un-stimulated control was increased with the addition of SLPI. Data was collected from 3 time points (4, 8 and 12 hour equivalent to n=1). All data were compared to untreated Hec-1A cells (control) from the relevant time point as control, given a nominal value of 1, mean ± s.e.m.

8.3.4 The effect of MMP-7 upon the expression of elafin mRNA in Hec-1A cells treated with inflammatory stimuli, TNFα + IL-1β

In order to further understand the possible interactions between elafin and MMP-7 the treatment of Hec-1A cells with MMP-7 protein concurrently with inflammatory stimuli, TNFα + IL-1β was undertaken.

Elafin mRNA, figure 8.3.4.1, was significantly increased in response to the addition of MMP-7 ($n=4$; $P<0.001$). The expression of elafin mRNA was over 12-point greater in cells treated with TNFα + IL-1β in the presence of MMP-7 over cells treated in the absence of MMP-7 (blue).

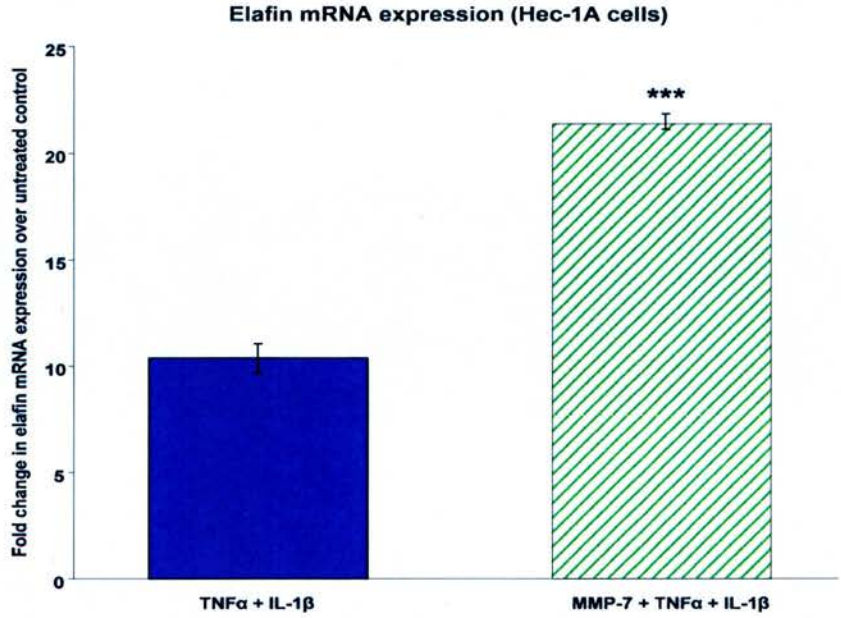


Figure 8.3.4.1 The effect of MMP-7 protein (5 ng/ml) on the expression of elafin mRNA (Hec-1A cells) in response to treatment with TNFα + IL-1β (n=4). Elafin mRNA was 12-point greater in the presence of MMP-7 (green) than without MMP-7 ($P<0.001$). Data was collected from 3 time points (4, 8 and 12 hour equivalent to n=1). All data were compared to untreated Hec-1A cells (control) from the relevant time point as control, given a nominal value of 1, mean ± s.e.m.

8.3.5 SLPI on the elafin promoter

In order to investigate the possibility that SLPI protein exerts its inhibitory effect upon elafin mRNA by direct interaction with the elafin promoter, Hec-1A cells were transiently transfected with pCAT reporter constructs (section 2.5, chapter 2). If SLPI binds to the elafin promoter, then the expression of the CAT reporter protein would be inhibited.

At the time of writing it has not been possible to obtain a sufficient level of cell viability in order for the results to be reliable.

Summary of results

1. SLPI protein inhibits the mRNA expression of elafin (figure 8.3.1.1) and MMP-7 (figure 8.3.1.2), in Hec-1A cells. SLPI acting in an anti-inflammatory capacity.
2. SLPI protein increases the mRNA expression of SLPI, hBD2 and hBD3 (figure 8.3.2.1, A-C) in Hec-1A cells. These data suggest a pro-inflammatory role for SLPI.
3. SLPI protein was found to increase the level of mRNA for the inflammatory cytokines TNFα and IL-1β (figure 8.3.3.1 A and B). An increase in the presence of these molecules would give rise to the increase observed in 2 above.
4. However, SLPI protein was shown to decrease the expression of IL-8 and IL-6 mRNA, both of these cytokines are associated with pro-inflammatory roles.
5. Treatment of Hec-1A cells with MMP-7 protein increased the level of elafin mRNA.
6. Attempts to identify an interaction between SLPI protein and the elafin promoter were inconclusive due to poor cell viability.

8.4 Discussion

Investigations that were undertaken in this thesis gave rise to a number of observations surrounding the possible interaction(s) between the molecules SLPI, elafin and MMP-7. Throughout the current thesis there were a number of results that were consistently suggestive of a relationship between the aforementioned molecules. In brief, the maximal expression of elafin and SLPI mRNA were found to be different when measured at different time points. This expression pattern appears to be reciprocal in fashion, with high elafin coinciding with lowered SLPI and high SLPI coinciding with lowered elafin (chapter 3). It has further been observed that the expression of SLPI and elafin occurs at different stages of the menstrual cycle, both in chapter 5 of the present thesis and as published previously (King, Morgan et al. 2003). This different expression pattern across the menstrual cycle was proposed to be due to the changing levels of the sex steroids and specifically progesterone (King, Morgan et al. 2003). SLPI has been shown to be upregulated by progesterone (King, Morgan et al. 2003) and this was confirmed in chapter 3 of the current thesis in the Hec-1A cell line. SLPI contains a progesterone response element (PRE) which enables this direct progesterone mediated response; thus, the maximal expression of SLPI is in mid-secretory endometrium. However, elafin has been shown to be maximally expressed during the menstrual phase, when circulating levels of progesterone are low (King, Critchley et al. 2003), chapter 5 of the current thesis. In contrast to SLPI however, there are is no PRE, thus any progesterone mediated effect is likely to be indirect (King, Morgan et al. 2003). The expression of elafin during the menstrual phase led to the theory that progesterone was likely to be inhibitory. Previous investigations using the breast cancer cell line

T47D, concluded that there was no effect upon elafin expression in response to treatment with progesterone in vitro (King, Morgan et al. 2003). However, in the current thesis, treatment of the endometrial epithelial cell line, Hec-1A with progesterone gave rise to an increase in expression of elafin (chapter 3). This led to investigations into the possible mechanisms of indirect progesterone mediation. Consideration was given to the fact that the treatment of Hec-1A cells with inflammatory mediators such as TNFα + IL-1β, in the presence of progesterone, was not an accurate representation of the endometrial environment. Primary endometrial explants are composed of different cell types, thus, possible paracrine influences upon the response to progesterone were investigated in chapter 4. In chapters 3 and 4 it was found that the expression of elafin was increased in the presence of progesterone, however, the expression of elafin was inhibited in the presence of stromal cells or stromal derived media in the presence of progesterone. Thus, the role for stromal factors in the epithelial expression of elafin was confirmed and further investigation of the literature identified MMP-7 as a possible stromal mediated factor. All three molecules, SLPI, elafin and MMP-7 formed the focus throughout the current thesis as a result. In chapters 6 and 7 it was shown that primary biopsy samples of Fallopian tube and intrauterine decidua collected from women in different groups had distinct patterns of expression for these genes. Thus, it was deemed necessary to explore the possibility of a relationship or interaction between these genes and in the time available a number of questions were addressed. The data obtained within the current chapter is summarised in figure 8.4.1.

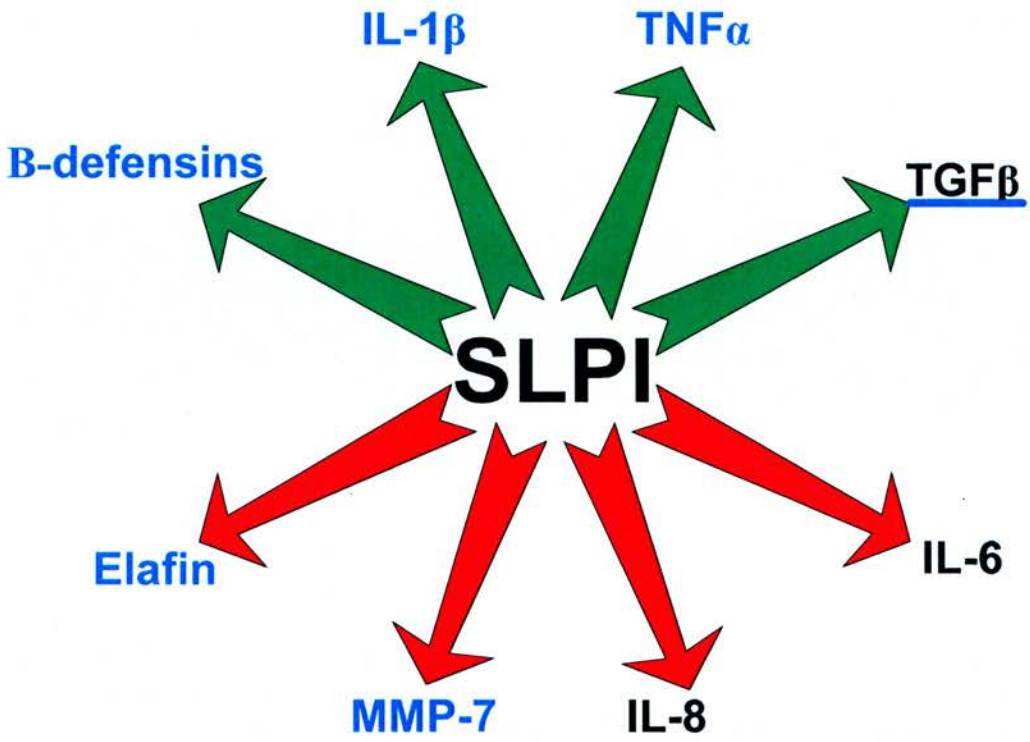


Figure 8.4.1 Summary of the effect of SLPI on the expression of the innate immune effectors investigated in this chapter. Green represents an increase and red a decrease. The molecules in blue are novel epithelial observations within the current thesis; the effect of SLPI upon the expression of IL-1β and TNFα has been reported in murine leukocytes (Lentsch, Jordan et al. 1999). IL-8 levels have been shown to be reduced in the lungs of cystic fibrosis patients in response to treatment with aerosolised SLPI (McElvaney, Nakamura et al. 1992). The expression of TGFβ was not directly investigated in this instance and has previously been reported to be upregulated by SLPI in murine macrophages (Sano, Shimizu et al. 2000).

The effect of SLPI protein upon the expression of elafin and MMP-7 was investigated in the Hec-1A cell line when stimulated with TNFα + IL-1β. SLPI was shown to significantly inhibit the expression of both elafin (figure 8.3.1.1) and MMP-7 mRNA expression (figure 8.3.1.2) in cells treated with inflammatory stimuli.

This effect may explain the observed differences in the expression of these genes as summarised in figure 8.1.3. Increased levels of SLPI may cause a decrease in the level of elafin and MMP-7, whilst lowered levels of SLPI may give rise to an increase in expression. In chapters 6 and 7 of the current thesis it was shown that elafin and MMP-7 mRNA levels were either both increased over SLPI or both decreased below the level of SLPI. To clarify, an instance where raised SLPI and MMP-7 with lowered elafin or an example of raised elafin and SLPI with lowered MMP-7 was not observed for any of the human Fallopian tube or decidua samples examined. Thus, it is reasonable to predict that some kind of interrelationship exists between these molecules, although the precise mechanism is beyond the scope of the current thesis. Any effect of SLPI upon MMP-7 and/or elafin may be direct or indirect. SLPI has been reported to be involved in the resolution of inflammation and has also been described as having a role as an anti-inflammatory molecule, with the precise mechanism still to be elucidated (Weldon, McGarry et al. 2007). The interaction of SLPI with anti-inflammatory mediators such as the cytokines IL-10 and TGFβ (Sano, Shimizu et al. 2000), may suggest an indirect means of action upon the expression of elafin and MMP-7. SLPI has been shown to increase the expression of TGFβ, which in this thesis has been shown to inhibit elafin mRNA in Hec-1A cells treated with inflammatory stimuli (chapter 3). The amount of IL-10 and TGFβ expression should be measured as part of future studies into the role of SLPI in Hec-1A culture experiments.

This effect of SLPI upon the expression of elafin and MMP-7 is in support of the anti-inflammatory nature of SLPI that has been proposed. However, when the effect

of SLPI was examined for other antimicrobials such as the human β-defensins (figure 8.3.2.1) the expression of hBD-2 and hBD-3 was found to be increased. It was also shown that SLPI mRNA is increased in the presence of SLPI. Thus, this anti-inflammatory action would appear to not be a generalised effect and an examination of the effects upon the pro-inflammatory cytokines TNFα and IL-1β (figure 8.3.3.1) offered some explanation. The increase in the level of these cytokines would in turn increase the expression of SLPI and the hBDs. The addition of SLPI with and without inflammatory stimuli brought about an increase in the level of hBD-2 and hBD-3. In order to investigate further whether the effect of SLPI upon hBDs is indirectly mediated by the increase in pro-inflammatory cytokines an experiment to block the expression of TNFα and IL-1β would be useful.

It was found that SLPI was inhibitory in the expression of the cytokines IL-8 and IL-6 (figure 8.3.3.2), which have been described as being inflammatory 'alarm' effectors of innate immunity. SLPI has been previously shown to inhibit the expression of IL-8 in LPS induced human monocyte-derived macrophages (Taggart, Cryan et al. 2005) and to inhibit IL-6 in human monocytes (Greene, McElvaney et al. 2004). This would lend credence to the role of SLPI as an anti-inflammatory protein.

One of the main mechanisms proposed for SLPI functioning as an anti-inflammatory mediator is via the interaction with the NF-κB pathway. It has been shown that the presence of SLPI leads to decreased activation of NF-κB in the lungs and this was associated with increased levels of the NF-κB inhibitor, IκBβ (Lentsch, Jordan et al. 1999). It has also been suggested that SLPI exerts effect upon the expression of pro-

inflammatory cytokines such as TNFα and IL-8, via direct competitive binding of the NF-κB-binding sites in the promoter regions of these genes (Taggart, Cryan et al. 2005). The interaction of SLPI with the NF-κB pathway in the modulation of the immune response, led to further consideration of a role for SLPI in the modulation of elafin expression. Elafin has also been shown to be regulated at the transcriptional level by NF-κB (Bingle, Tetley et al. 2001) in the epithelial cells of the lung. However, it has also been proposed that the transcriptional activation of elafin is cell specific, as in mammary epithelial cells it is the transcription factor activating protein-1 (AP-1) that regulates activation (Zhang, Zou et al. 1995). Thus, it is conceivable that the ability of SLPI to interact with transcription factors and the expression of elafin being modulated by the presence of an NF-κB site within its promoter is a good route of interaction between these two molecules at the transcriptional level. The provision of plasmid constructs containing the elafin promoter attached to a CAT reporter gene enabled some investigation into this possibility.

However, despite repeated attempts, it has not been possible to maintain the viability of cells subsequent to both the transfection and stimulation with IL-1β + TNFα. The cells were successfully transfected with β-gal reporter constructs and cell viability was maintained, thus, it was not thought to be the process of transfection affecting the cells. The untreated controls where viable and this has led to the conclusion that it is the subsequent treatment with pro-inflammatory cytokines that was affecting the viability of the cells. Further work is under way to determine the optimal time post transfection to stimulate the cells prior to lysis and protein measurement.

Preliminary analysis of the possible relationship between MMP-7 and elafin was undertaken and it was shown that the addition of MMP-7 to Hec-1A cells concurrent with inflammatory stimuli gives rise to an increase in the level of elafin mRNA (figure 8.3.4.1). The apparent 'coupled' expression of both elafin and MMP-7 has been demonstrated in the Fallopian tube (chapter 6) and in the endometrium (chapters 5 & 7). Therefore it is possible that MMP-7 has a role in the regulation of expression of elafin. As has been described previously elafin is transcribed as pre-elafin (trappin-2), which is thought to undergo proteolytic cleavage in order to release the elafin molecule (Guyot, Zani et al. 2005). The mechanism or factors involved in the cleavage of pre-elafin into elafin has yet to be fully elucidated. The proteolytic cleavage of pre-elafin by tryptase has been suggested by Guyot et al (Guyot, Zani et al. 2005) in the lungs of patients with cystic fibrosis (CF). Guyot et al. also undertook to investigate the metalloproteases as candidates for the processing of pre-elafin and showed that there was no activity against either pre-elafin or elafin (Guyot, Zani et al. 2005). However, their study was restricted to that of MMP-8 and MMP-9 due to the abundance of these respective molecules in the lung and the established proteolytic role of these enzymes. Thus, the possibility was considered as part of this thesis, that the high level of MMP-7 expression within the female reproductive tract and the observed associative expression with elafin, may implicate such a role. The role of MMP-7 could be either with the release of the elafin molecule via the cleavage of pre-elafin or it could also serve to degrade elafin. It is also possible that elafin serves to degrade excess MMP-7, in order to modulate the activity of this enzyme during mucosal repair.

It is clear that the function of natural antimicrobials within the innate immune system is still to be fully determined and further characterisation of their role within the female reproductive tract would offer opportunities for a greater understanding of this locally regulated defence system. The female reproductive tract is a unique environment in which the cyclical injury and repair process, hosting of non-self sperm and the semi-allogeneic foetus, which involves complex immune regulation. Thus, there are also opportunities to explore the potential for the female reproductive tract as a model system in order to fully understand and implement the findings in other mucosal systems in an attempt to control inflammatory disorders.

8.4.1 Future work

The aim of this chapter was to address some of the questions that have been raised through the course of this thesis. However, due to time constraints it was only possible to address a few of the possibilities as presented. The effect of SLPI protein on the expression of elafin, MMP-7 and the hBDs was explored and served to suggest a role for the interaction between antimicrobial proteins. Thus, it would be useful to identify the effect of other antimicrobials upon the expression of innate immune effectors in a similar fashion to that presented for SLPI. In order to further understand the interaction between elafin and SLPI, it is necessary to undertake experiments involving the treatment of cells with recombinant elafin. The use of anti-SLPI in culture experiments would also be useful to in determining the effects of SLPI on the expression of elafin and other immune effectors.

The effect of MMP-7 upon the expression of SLPI and the other natural antimicrobials should be investigated in order to further determine any role for MMP-7 in their modulation. The possibility of MMP-7 having a role in the processing of pre-elafin and other antimicrobials within the female reproductive tract also merits further investigation. Preliminary attempts at cleaving pre-elafin with MMP-7 in collaboration with colleagues in the Centre for Inflammation Research (Dr Paul Fitch, University of Edinburgh) have proved inconclusive and form part of ongoing studies. The effect of elafin protein on that of MMP-7 should also be investigated as it is possible that elafin is responsible for the degradation of the metalloproteases.

The interaction between elafin, SLPI and MMP-7 is likely to be indirect through the modulation of other innate immune effectors and via transcription pathways. Thus, a systematic analysis of the signalling pathways and identification of the mechanism of interaction is required.

Chapter 9:

General Discussion

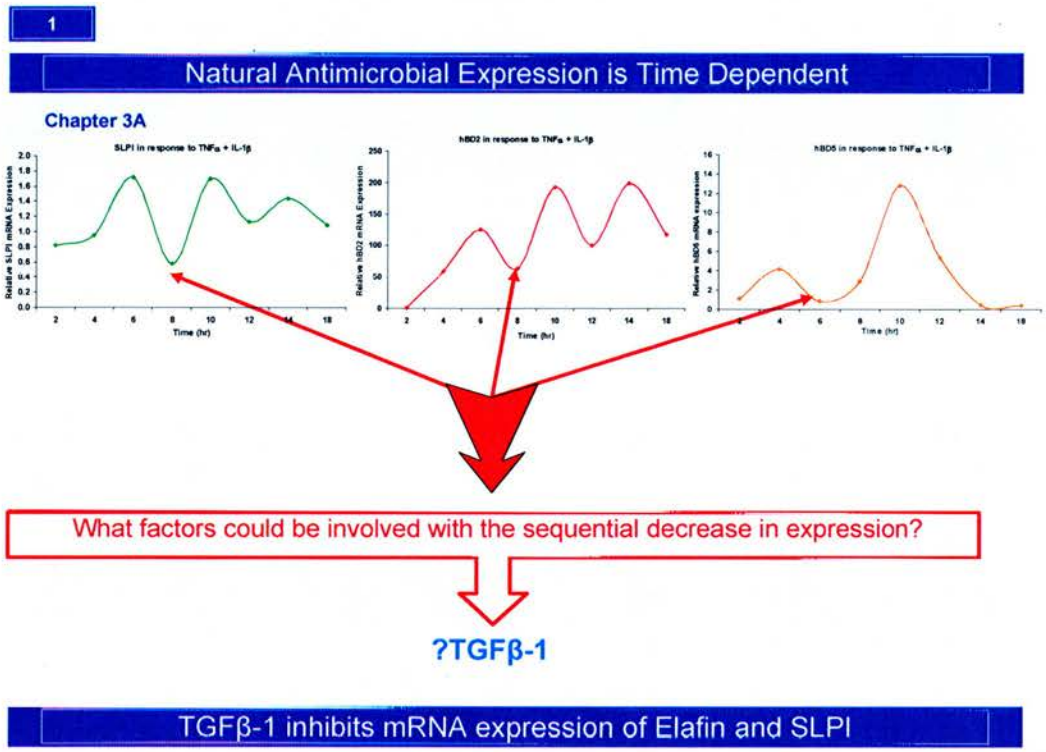
9.1 Synopsis of Results

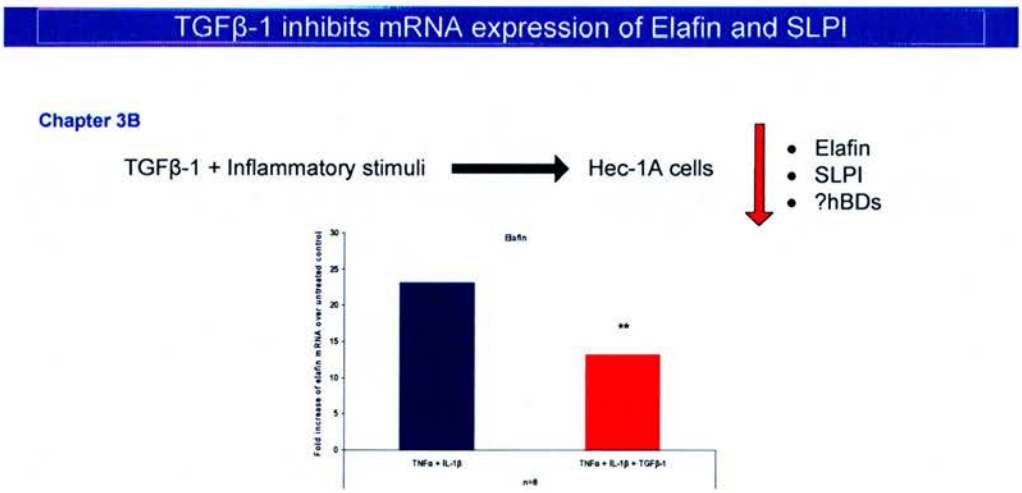
The aims of this thesis have been to characterise further the expression and regulation of natural antimicrobials in the female reproductive tract. The investigations were undertaken using the Hec-1A endometrial epithelial cell line, stromal cells cultured from endometrial explants (primary cell culture) and tissue collected from women undergoing gynaecological procedures: Fallopian tube, endometrium and first trimester decidua. As a result of the investigations undertaken in this thesis the role of other innate immune effectors was also considered. The roles of MMP-7 and TGFβ-1 were investigated as it became apparent that these proteins may have a role in the expression and/or modulation of natural antimicrobials.

The data in this thesis have been presented in distinct chapters and discussed in depth within the focus of each chapter and the context of the motivations that dictated the investigations undertaken. However, as the work progressed it was apparent that there was considerable overlap in both the observations and the questions raised as a result of the data obtained. The purpose of this chapter is to provide an overall synopsis of some of the main points for discussion of the thesis as a whole and to allow discussion in a wider context.

The main points and data are presented in 5 schematic summaries and discussed within the context of each of the summaries.

9.1.1 Summary of results

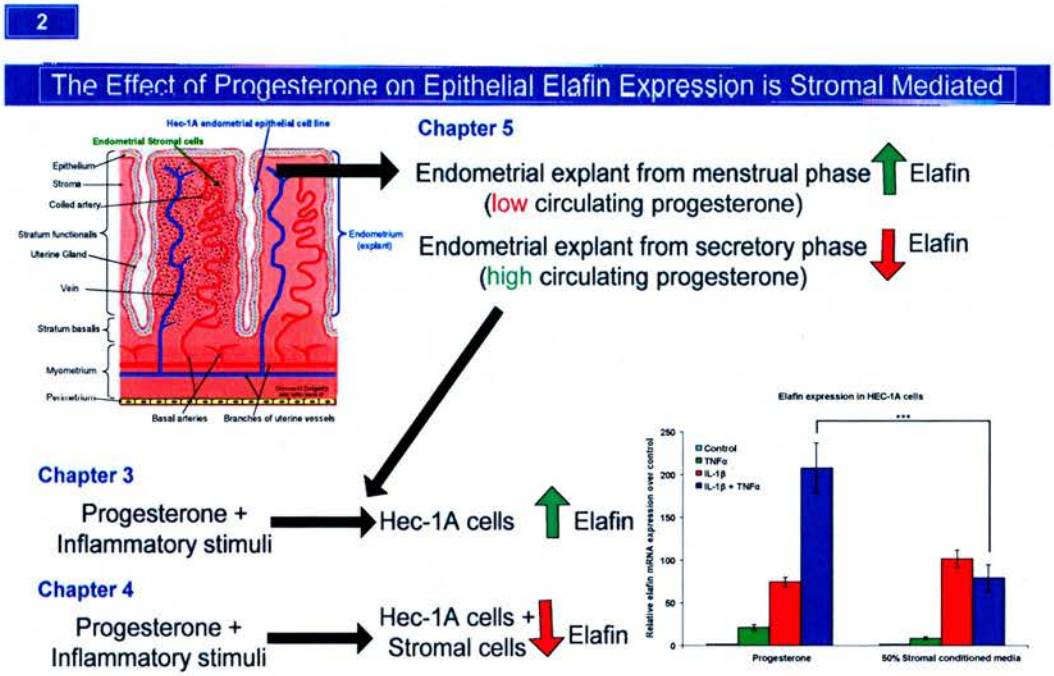




The treatment of the endometrial epithelial cell line, Hec-1A with pro-inflammatory cytokines, IL-1β + TNFα resulted in the time dependent expression of natural antimicrobials. The phasic pattern of expression exhibited by the natural antimicrobials is differential for each of the antimicrobials that were investigated.

Peak expression levels were followed by a rapid decrease in the level of expression and this led to considerations of factors involved in this decrease. The growth factor, TGFβ-1 was investigated as a candidate factor in the decrease in expression of natural antimicrobials. This was in part motivated by reports in the literature that SLPI is decreased in the presence of TGFβ-1 (Jaumann, Elssner et al. 2000). It was shown that TGFβ-1 can also inhibit the expression of elafin and SLPI mRNA in the Hec-1A cell line.

Future work would involve the exploration of the effect of TGFβ-1 upon the expression of other natural antimicrobials; preliminary data suggests that some of the β-defensins may be similarly inhibited. It would also be useful to explore the relative levels of TGFβ-1 in the culture media of the time course experiments to determine whether any fluctuation in expression could be attributed to the phasic decrease in expression observed. A consideration of other factors that may bring about the decreased expression of natural antimicrobials observed during time course experiments would also be useful. Further consideration of other innate immune effectors such as the anti-inflammatory cytokine, IL-10 is also necessary in order to further understand the regulation of natural antimicrobials.



It was previously shown that elafin is maximally expressed in endometrial samples collected from the menstrual phase of the cycle when circulating levels of progesterone are low (King, Critchley et al. 2003) and chapter 5). This was in contrast to the data obtained from treatment of the Hec-1A cell line, where the addition of progesterone increased the expression of elafin. The role of paracrine mediated factors was investigated and the presence of stromal cells in the treatment of epithelial cells with progesterone and inflammatory stimuli inhibited the expression of elafin. Treatment of stromal and epithelial cells without the presence of progesterone increased the epithelial expression of elafin over that for epithelial cells alone. Suggesting that there is a role for stromal cells in the epithelial expression of elafin and this merits further investigation in terms of the innate immunity of the endometrium. However, the effect of progesterone appeared to be

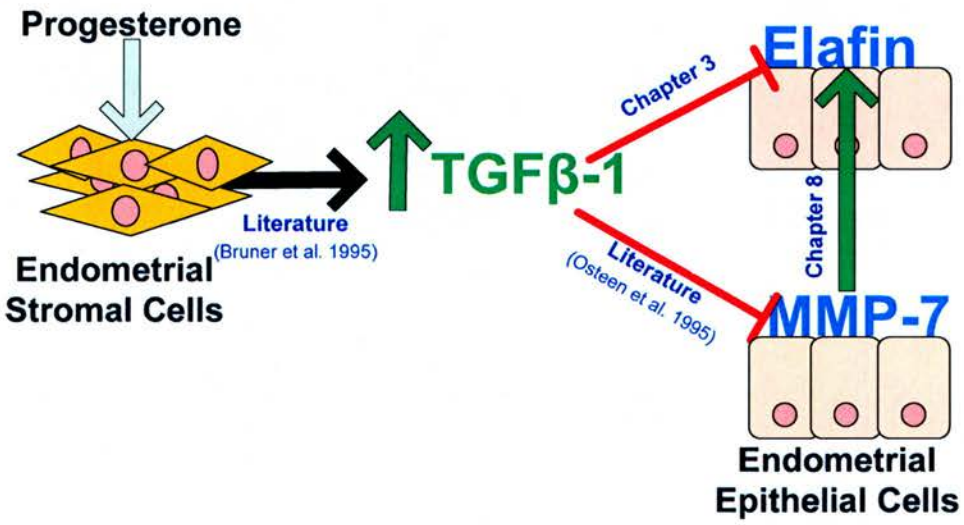
stromal mediated and offered an explanation for the apparent differences in expression between primary material and the cultured cell line. This is discussed further in part 3 below.

Future work, the effect of stromal mediated factors upon the expression of other natural antimicrobials such as SLPI and the hBDs is required. Human β-defensin 2, as with elafin has been similary shown to be maximally expressed in endometrial samples collected during the menstrual phase (low circulating levels of progesterone) (King, Critchley et al. 2003). In chapter 3 of the current thesis the treatment of Hec-1A cells with TNFα + IL-1β in the presence of progesterone inhibited the expression of hBD2 mRNA (Chapter 3B, figure 3.8.3.3). The use of a progesterone antagonist, RU486, was also utilised in chapter 3. The addition of RU486 and progesterone to Hec-1A cells gave rise to a further increase in the level of elafin mRNA over the previous increase in the presence of progesterone (figure 3.8.3.2; chapter 3B). Whilst hBD2 levels were increased in contrast to the previous inhibition observed in the presence of progesterone alone (figure 3.8.3.3; chapter 3B). The use of anti-progestins in the co-culture set-up would be useful in furthering the understanding of the role of progesterone both directly and indirectly.

3

Stromal Mediated Candidate Factors: TGFβ and MMP-7

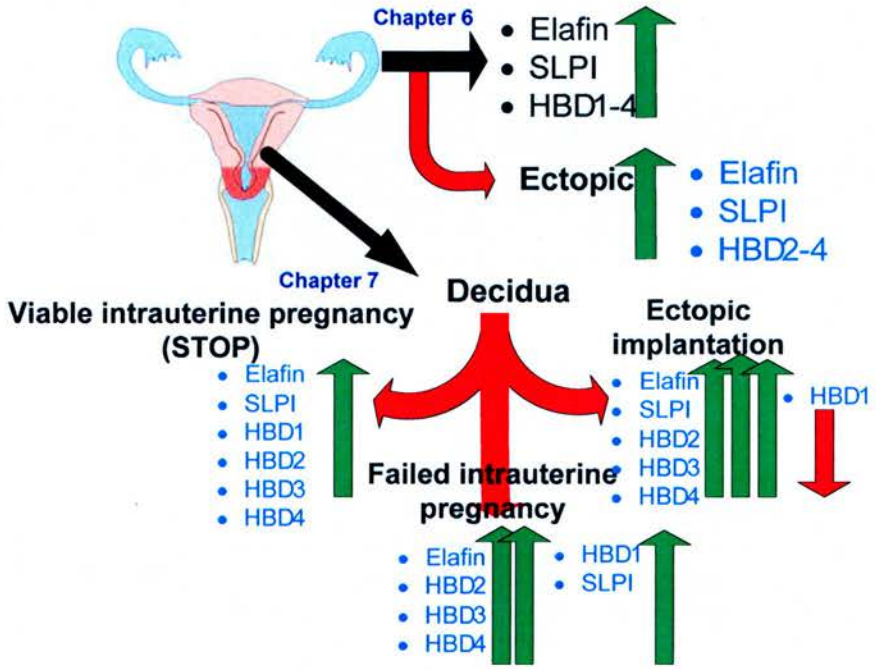
Chapter 4



Following on from the identification of a role for the stroma in the epithelial expression of elafin the factors and possible mechanisms of this mediation were explored. Having already identified that TGFβ-1 has an inhibitory effect upon the expression of both elafin and SLPI, this was investigated further. Endometrial stromal cells upregulate the expression of TGFβ-1 in response to progesterone (Bruner, Rodgers et al. 1995) and this in turn has been reported to mediate the epithelial expression of MMP-7 (Bruner, Rodgers et al. 1995; Osteen, Keller et al. 1999). In this thesis it was shown that MMP-7 could increase the expression of elafin mRNA. This may suggest that an increase in TGFβ-1 from the stroma in response to progesterone could result in the inhibition of elafin by both directly or indirectly through the inhibition of MMP-7 as shown above.

4

Natural Antimicrobial Expression in the Female Reproductive Tract

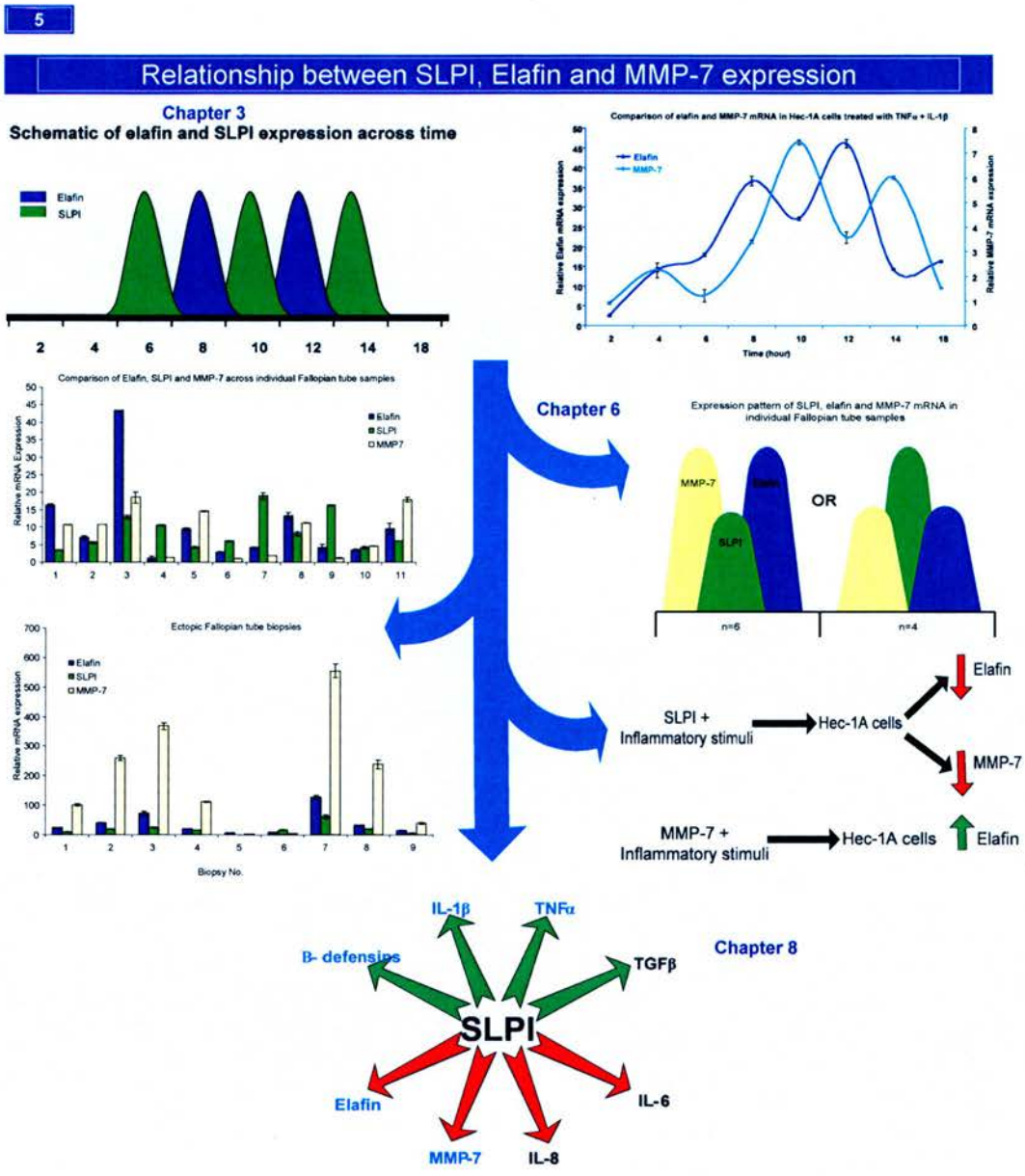


An examination of natural antimicrobial expression in the human Fallopian tube confirmed the expression of SLPI, elafin and hBD1-4 at this site of the female reproductive tract. Furthermore there was a preliminary indication that this expression was hormonally regulated across the ovarian cycle as was observed previously in the human endometrium (King, Critchley et al. 2003). Fallopian tube biopsies collected from women with an ectopic gestation were also investigated for the expression of natural antimicrobials. The presence of an ectopic gestation increased the expression of SLPI, elafin and hBD2-4, whilst hBD1 remained unchanged. The increased expression of natural antimicrobials in the event of an ectopic pregnancy could offer an insight into the inflammatory mechanisms

involved. However, further work is required in order to establish the difference between a causal and a consequential involvement for natural antimicrobials.

Further investigations were undertaken with biopsy samples obtained from the decidua of women undergoing surgical management for ectopic pregnancy for the expression of natural antimicrobials. This was compared with samples obtained from women undergoing surgical management for miscarriage and for the termination of a viable intrauterine pregnancy. With the exception of hBD-1, all antimicrobials (hBD2-4, SLPI and elafin) were found to be increased in the decidua obtained from an ectopic gestation. It was further identified that there was a differential level of expression when compared with samples obtained from intrauterine failed early pregnancy (miscarriage) and from viable intrauterine pregnancy (STOP). This differential pattern of expression may present opportunities in establishing a method of diagnosis, where an ectopic pregnancy could be distinguished from a miscarriage, by study of a decidual sample collected from the uterine cavity.

In addition to an analysis of natural antimicrobial expression in the tissue resource described above (4), the expression of MMP-7was also examined. An interesting pattern of associative expression was observed between elafin, MMP-7 and SLPI and this is discussed in the context of other results in the next section.



The expression of natural antimicrobials has been described as being time dependent and that the timing of SLPI and elafin expression is reciprocal, when elafin expression was increased this corresponded with lowered expression of SLPI and vice versa. This pattern of expression was thought to be an indication of possible interaction between these two anti-proteases and was investigated further.

The identification of MMP-7 as a possible paracrine mediator in chapter 4 and the observation of a similar level of differential time dependent pattern of expression in chapter 8 (figure 8.1.1) when compared to elafin, motivated further study. The pattern of MMP-7 mRNA exhibited was similar to that observed for elafin, but with a 2 hour lag in time. MMP-7 maximal peaks in expression were observed to occur subsequent to the maximal peaks in expression of elafin. Elafin was observed to peak after 8 hr and a 2nd greater peak occurred at 12 hr, whilst MMP-7 showed the largest peak at 10 hr and a 2nd smaller peak at 14 hr. It may be that MMP-7 is involved in the splicing of pre-elafin into elafin, or perhaps to remove elafin to prevent too great an immune response.

A comparative examination of the expression of elafin, SLPI and MMP-7 also appeared to reveal a specific pattern in biopsy samples obtained from the Fallopian tube and early pregnancy decidua. In the samples investigated one of two expression scenarios were consistently found to be demonstrated: Either increased SLPI greater than that observed for both elafin and MMP-7 OR increased elafin and MMP-7 greater than for SLPI. Although the precise mechanism involved is unclear it does appear that some kind of relationship between these three molecules exists and further investigation may offer both explanation and opportunities for a greater understanding.

Biopsy samples of the decidua and Fallopian tube obtained from women with an ectopic gestation were found to have a significantly increased level of MMP-7 mRNA with a corresponding but lower increase in elafin and SLPI. The significance of this elevated expression in MMP-7 and the apparent deviation from the pattern

observed in normal tissue needs further investigation. An exploration into the cause or effect of increased expression of MMP-7 is required.

An investigation into the role of SLPI upon the expression of other innate immune effectors was commenced in chapter 8 of the current thesis. SLPI was found to decrease the expression of both elafin and MMP-7, whilst increasing the expression of defensins and the inflammatory cytokines TNFα and IL-1β. Further work is required in order to understand the mechanism by which SLPI exerts these effects, especially the relationship between elafin and SLPI.

9.2 General Discussion

The work in this thesis was undertaken in order to further understanding of natural antimicrobial expression and their interactions within the innate immune system of the female reproductive tract. These studies also offer some answers but, inevitably with research the thesis gave to rise to further questions. It is clear that the expression of natural antimicrobials is rapid and short term, whilst the factors involved in the upregulation are well understood those involved with the sudden and rapid decline are not so clear. The importance of understanding the modulation of natural antimicrobials is due to the translational opportunities that would arise from the establishment of effective control (Bowdish, Davidson et al. 2006). The control of expression would enable both the prevention of a destructive inflammatory response and as well as increasing protection when required. Furthermore it should be considered that there is an increasing concern with regard to microbial resistance to our current synthetic repertoire of antibiotics (Wright 2007). Thus, there is a growing interest in the development of natural products to fulfil the role in the treatment of infections, and the natural antimicrobial peptides offer such possibilities (McPhee and Hancock 2005).

Within the female reproductive tract it has been shown that the natural antimicrobials are subject to hormonal regulation (King, Critchley et al. 2003) and this has also been shown to be an important consideration with regard to the use of contraceptives (Fleming, King et al. 2003). In this thesis it has been shown that elafin is likely to be affected by progesterone in an indirect manner through interaction with other factors

and cells. This requires to be further investigated for other natural antimicrobials and in relation to the effect upon other cells and inflammatory mediators. The role of sex hormones in the regulation of innate immune effectors is important in order to understand the consequences of both systemic and local hormone levels upon immune status.

The demonstration in chapter 3 that the natural antimicrobials were differentially expressed in a time dependent manner and that different peptides were expressed at different times suggested distinct roles and or interactions. There also seemed to be a level of associative expression between some of the molecules, such as being expressed together (same timepoint) or in opposition to one another. The potential for synergistic action is a likely explanation for this pattern of expression and indeed other groups have shown antimicrobials act in a synergistic manner, for example SLPI in the lung (Singh, Tack et al. 2000). It has also been demonstrated that a synergistic relationship exists between hBD2 and LL-37 (Dorschner, Lin et al. 2003).

The expression of natural antimicrobials in the human Fallopian tube was established within this thesis. There was also a suggestion that this expression may be affected by the ovarian cycle and this needs to be further investigated in order to fully understand the role of natural antimicrobials within the Fallopian tube. The expression of natural antimicrobials and the potential modulation of other immune factors and the effect upon the mucosal and cellular environment may also reveal the function of the immune response within the Fallopian tube. In the Fallopian tube biopsies collected from women with an ectopic gestation it was shown that there was

an increase in natural antimicrobial expression. There was also a large increase in the level of MMP-7 which may suggest a role for this matrix metalloprotease protein (MMP) in the event of an ectopic pregnancy. The role of MMPs in the human Fallopian tube is poorly understood and their involvement in the repair of the extra cellular matrix (ECM) raises the possibility of a role in implantation (Bai, Wang et al. 2005). The high level of MMP-7 may indicate an increased risk of an ectopic implantation, as high levels of MMP-7 would cause an increased immune response and lead to scarring in the Fallopian tube. The role of MMP-7 within the female reproductive tract requires to be investigated further. MMPs have been implicated in the pathogenesis of Fallopian tube damage in response to infection (Ault, Kelly et al. 2002). The excessive degradation of the ECM and subsequent scarring of the Fallopian tube would impede the progress of the conceptus. However, the increased expression of both natural antimicrobials and MMP-7 may also be likely as a result of the inflammatory nature of an ectopic gestation and further work into the cause or effect, and the roles of these molecules is merited.

The decidua from women with an ectopic gestation was also investigated and compared alongside decidua biopsies obtained from the surgical management of miscarriage and termination of first trimester pregnancy. The expression of natural antimicrobials and MMP-7 in the decidua from an ectopic gestation was representative of the data observed in corresponding Fallopian tube biopsies. As was discussed in chapter 7 this raises the possibility of natural antimicrobials being utilised as molecular markers or in the early diagnosis of an ectopic pregnancy through sampling of uterine decidua.

The data in this thesis have raised many questions and perhaps really only offers a prelude to the possibilities for a greater understanding of the innate immune effectors and involvement in the function of the reproductive tract.

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Appendix 1: General Materials

Tissue Collection and Culture	Source
Pipelle suction curette	Pipelle, Laboratoire CCD, France
RPMI 1640 medium	Sigma, Poole, Dorset, UK
Neutral Buffered Formalin (NBF)	See appendix 2
Complete RPMI	See appendix 2
F supplement	See appendix 2
Phosphate Buffered Saline (PBS)	Sigma
Ethanol	Hayman Ltd., Essex, UK
Collagenase	Sigma
DNAase	Sigma
Matrigel	Sigma
Fetal Calf Serum (FCS)	Myoplex, PAA Laboratories, UK
Penicillin	Sigma
Streptomycin	Sigma
Gentamycin	Sigma
L-Glutamine	Sigma
Transwells	Costar, High Wycombe, UK
Culture flasks and plates	Costar, High Wycombe, UK
Cell Line	**Source**
Hec-1A	American Type Culture Collection, Manassas, VA)
Treatments and supplements	**Source**
Oestradiol	Sigma
Basic fibroblast growth factor (bFGF)	Peprotech
Progesterone	Sigma
RU 486 (mifepristone)	Sigma
Lipoteichoic acid (LTA) *S. aureus*	Sigma
Lipopolysaccharide (LPS) *E. coli*	Sigma
Interleukin-1β (IL-1β)	Peprotech
Tumour Necrosis Factor α (TNFα)	Peprotech

Transforming Growth Factor β-1 (TGFβ-1)	Peprotech
Matrilysin (MMP-7)	Sigma
SLPI	Sigma
Anti-SLPI polyclonal	Hycult

RNA Extraction	**Source**
Total RNA Isolation Reagent (TRIR)	Abgene Limited, Epsom, UK
Chloroform	BDH Laboratory Supplies, UK
Isopropanol	Sigma-Aldrich Co. Ltd.
Ethanol	Hayman Ltd., Essex, UK
RNA storage solution	Ambion Inc, Austin, Texas, USA
Phase Lock Gel Eppendorfs	Eppendorf AG, Hamberg, Germany
TE Buffer	See appendix 2
RNA storage solution	Ambion
RNeasy Mini Kit	Qiagen, UK

RT-PCR	**Source**
cDNA reagents	PE Biosystems, Warrington, UK
Mineral Oil	Sigma
PCR Master mix kit	Applied Biosystems
18s primer/probe kit	PE Biosystems, Warrington, UK
Primers and probes	Biosource, Nivelles, Belgium

Immunohistochemistry	**Source**
Acetone	BDH Labotatory supplies
PBS	See appendix 2
PBS + Tween	See appendix 2
0.01M Sodium Citrate	See appendix 2
Hydrogen peroxide	BDH Laboratory supplies
Goat serum	Vector Laboratories, UK
Protein Block	Vector Laboratories, UK
Avidin block	Vector Laboratories, UK
Biotin block	Vector Laboratories, UK
Avidin-biotin-peroxidase complex (ABC)	Vector laboratories, UK

Diaminobenzidine (DAB)	Vector laboratories, UK
Harris' hematoxylin	Pioneer Research Chemicals Ltd. UK
Xylene	BDH Laboratory Supplies
Pertex	Cellpath plc, Hemel Hempsted, UK

ELISA	**Source**
96 well plates	Nunc Maxi-Sorp, Gibco, UK
Wash buffer	See appendix 2
Carbonate buffer	See appendix 2
Blocking solution	See appendix 2
Streptavidin peroxidase	Sigma
Substrate	See appendix 2
2N Sulphuric acid	Sigma
CAT ELISA kit	Roche
SLPI ELISA kit	R&D Systems

Appendix 2: Recipes for solutions

All chemicals listed were from Sigma and all dilutions were in distilled water unless otherwise stated

Blocking solution

In 1 litre: 20g 2% polyvinylpyrrolidone
5g bovine serum albumin
1ml preservatives
1.9g EDTA (5 mmol/L)
6.1g Tris (50 mmol/L)

Boehringer Mannheim

B-galactosidase assay solutions

Wash buffer pH7.3 (1000 ml):
46 ml 0.5M-NaH_2PO_4
154 ml 0.5M-Na_2HPO_4
2 ml 1M-magnesium chloride
10 ml 1% (w/v) deoxycholate
10 ml 2% (v/v) nonidet-P40
Final volume with ROP H_20 to 1000 ml

Fix solution pH 7.3 (200 ml):
9.2 ml 0.5M-NaH_2PO_4
30.8 ml 0.5M-Na_2HPO_4
1.6 ml 25% (w/v) gluteraldehyde
10 ml 100 mM-EGTA; pH7.3
0.4 ml 1.0 M-magnesium chloride
Final volume 200 ml with ROP H_2O

X-gal stain (250 ml):
11.5 ml 0.5M-NaH_2PO_4
38.5 ml 0.5M-Na_2HPO_4
0.53 g Potassium ferrocyanide
0.41 g Potassium ferricyanide
2.5 ml 1% (w/v) deoxycholate

2.5 ml 2% (v/v) nonidet-P40

0.5 ml 1M-magnesium chloride

Final volume 250 ml with ROP H_2O

Complete medium (cRPMI)

500ml RPMI 1640 supplemented with

10% FBS

20μg/ml gentamycin

100 IU/ml penicillin

100μg/ml streptomycin

2 mM L-glutamine

ELISA buffer

In 1 litre: 9g NaCl (150mmol/L)

12.1g Tris (100mmol/L)

300μl phenol red solution (0.00015%) Flow Laboratories, UK

0.7g EDTA (2mmol/L)

150mg 2-methylisothiazolone (1mmol)

150mg bromonitrodioxane (1mmol)

2g BSA

pH 7.2

ELISA substrate

1ml tetramethyl benzidine : 1ml urea-hydrogen peroxidase : 10ml sodium acetate

5g/l urea-hydrogen peroxidase (0.5% in 50mM sodium acetate, pH6)

3g/l tetramethyl benzidine in dimethylformamide

in 100mmol/l sodium acetate, pH6

100mM sodium acetate

In 1 litre: 13.6g sodium trihydrate

1ml preservatives

pH 6

ELISA wash buffer

In 1 litre: 9g NaCl (150mmol/L)
1.21g Tris (5mmol/L)
0.5ml Tween-20 (0.025%)
pH 7-7.5

Neutral buffered formalin (NBF)

In 1 litre:		
	6.5g Na_2HPO_4	BDH
	4.5g $NaH_2PO_4.2H2O$	BDH
	100ml 40% formaldehyde	
	900ml distilled water	

Phosphate buffered saline (PBS)

In 1 litre: 5 PBS tablets
pH 7.4 – 7.6

PBS + Tween

In 1 litre: 5 PBS tablets
8g NaCl
100µl Tween-20
pH 7.4 – 7.6

Preservatives

200mg/ml 2-methylisothiazolone
200mg/ml bromonitrodioxane
in dimethylformamide (DMF) / dimethylsulphoxide (DMSO) 1:1
diluted 1:1000 to use

0.1M Sodium citrate buffer

In 1 litre:	29.4g Tri-sodium citrate	BDH
	0.1g sodium azide	
	pH 6	
	Diluted 1:10 to use	

TE Buffer

10mmol/l Tris pH8.0

1mmol/l EDTA in DEPC water

Tris buffer

In 1 litre:	121.1g Trizma base
	pH 7.2